SEPARATION BY UNIT GRAVITY SEDIMENTATION OF T-CELLS SENSITIZED TO SOLUBLE ANTIGEN

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1977

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I. Introduction

The immune system is a complex system involving several different cell types acting both as antigen recognizers and as effector cells, secreting antibodies or other substances or acting directly. It is traditional to divide the immune system into two parts: the secretion of antibody, provided by Bursa or Bursa-equivalent derived cells (B cells) and the cell mediated system composed of tymus derived cells (T cells). This separation is undoubtedly an oversimplification as many antibody responses require the assistance of T-cells, which are themselves not a homogeneous population of lymphocytes. This report will discuss T-cell function and describe a method of purification of active T-cells.

T-lymphocytes are known to be heterogeneous, both with respect to surface properties and function. Among the functions they serve are tumor immunity, immunity to viruses, allograft rejection, delayed-type hypersensitivity to soluble antigens and assistance in B-cell responses. They secrete soluble factors, such as lymphotoxin (LT), macrophage inhibiting factor (MIF) and many others, and can be directly cytotoxic and suppress the responses of various other cell populations.

Despite their numerous functions, T cells have not been as easy to study in vitro as B cells. The specialized products of T cells are secreted in small quantities and have most often been studied by biological assay instead of more direct biochemical means. Lymphocytes cannot be maintained in culture for long periods of time, and the tumor lines currently available are non-specific with respect to antigen, as well as differing from normal T cells in response to such things as mitogens.

The study of B-cells has been aided by the existence of myelomas which secrete large amounts of immunoglobulin. This allows the study of both the B-cell product and membranes of the clones of cells. B-cell study has also been facilitated by the existence of single cell techniques such as the plaque forming assay.

These problems have led to attempts to isolate specific classes of T-cells, either by making use of a membrane property (e.g. Ly antigen), an in vitro function (response to PHA) or a physical property (density). Experimenters attempt to obtain a population of cells that are enriched for some structural or functional attribute, in order to better study this complex set of cells.

In this study we show that it is possible to separate specifically sensitized T-cells from other lymph node lymphocytes on the basis of their increased size using the technique of unit gravity sedimentation. We use a system modified by Hecht et al (1976) from that originally described by Miller and Phillips (1968). We have isolated a population of T-cells greatly enriched in the T-cell that responds to a specific soluble antigen. Work is in progress to fuse this population with an already established T-cell lymphoma in order to create an antigen specific T-cell line.

II. T-cell heterogeneity and function

The in vivo response to stimuli that induce cell mediated immunity is complex and is probably the result of the interaction of both effector and control T-lymphocytes as well as macrophages. In order to separate out the components of this response, various cell markers have been used. There are several murine antigen systems which have been well

studied and have proved to be powerful tools in the investigation of the functions of populations of lymphocytes. These include the Thy antigen (formerly θ), MSLA and the Ly system. This last has been an especially useful tool in the study of T-cell function, allowing the study of such questions as: Are the various functions of T-cells properties of separate populations of cells distinguishable by cell surface antigens? Does one cell line differentiate into another or are the different cell lines the result of parallel lines of differentiation? Is the differentiation of cells influenced by exposure to antigen?

The Ly system contains several loci, three of which are relevant to T-cells, each with two alleles. Ly1 is on chromosome 19, and the closely linked Ly2 and Ly3 loci are on chromosome 6. These antigens are found only on thymocytes and lymphocytes. To date Ly2 and Ly3 have not been separated. Thus no cells with the phenotype $Ly2^+$ $Ly3^-$ or $Ly2^-$ $Ly3^+$ have been discovered. There are three classes of lymphocytes: $Ly1^+2^+3^+$ (=Ly123). $Ly1^+2^-3^-$ (=Ly1) and $Ly1^-2^+3^+$ (=Ly23). In the mouse approximately 50% of the peripheral T cells are Ly123, 33% ly1 and 5-10% Ly23.

It is not yet clear what role, if any, the Ly antigens play in cell function. Shiku et al (1976) found that antibody binding to the Ly antigens did not inhibit the cytotoxic functions of the cells, suggesting that in this system at least, the Ly antigens are not needed for antigen recognition and cell function.

Studies done by several groups involve assaying for a particular T-cell function (e.g. cytotoxicity or helper function) and comparing the activity of unseparated cells with a population of cells treated with an antiserum to one of the Ly antigens (perpared in syngeneic animals differing only at that Ly locus). To the extent that the antiserum destroys cells, the function is assumed to be a property of cells expressing that particular Ly antigen. In some cases one group of cells may suppress another. This will be seen as an increase in activity when the suppressing cells are removed with antiserum. To distinguish between the functions of Ly123 cells and those that require both Ly1 and Ly23, animals are depleted of all T-cells and combinations of Ly1 and Ly23 cells are added back to see if the activity can be restored. If it cannot, Ly 123 cells are assumed to be required.

The work of several groups has led to the conclusions presented in Table 1 (adapted from Cantor et al, 1976). From this we can see that T-cell functions can be separated on the basis of different cell membrane antigens. Helper function is the ability to help B cells produce a primary response to SRBC in vitro. Cytotoxicity refers to the specific killing by lymphocytes of cells to which the mouse had previously been sensitized. The cells may be either allogeneic or syngeneic. Perhaps somewhat surprisingly, Shiku et al (1976) found that while Ly23 cells mediate cytotoxicity to allogeneic cells, the phenotype of cell which mediate killing of syngeneic tumor cells are Ly 123. That is to say that both antisera to Ly1 and Ly23 eliminate syngeneic killing which cannot be restored by adding a combination of Ly1 and Ly23 cells. They cannot rule out the possibility that one of these cells is involved as well.

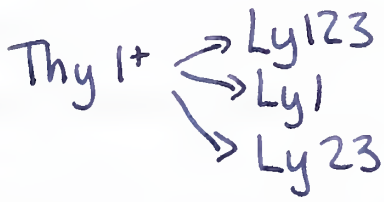
T-cell function	Lyl	Ly 23	Lyl23	Reference
Helper activity (T-B,T-T)				Cantor et al (1976)
Primary response	+		?	Cantor and Boyse (1975b)
Secondary response	+			Kisielow et al (1975)
Suppressor activity				
Primary		?	?	
Secondary		+ +	+	Cantor et al (1976)
Allotype suppression		+		"
Polyclonal induction		+		Jandinski et al (1976)
Killer activity				
Prekiller		+	?	
Effector:				
allogeneic		+		Cantor and Boyse (1975a)
syngeneic			+	Shiku et al (1976)
Mixed Leukocyte Cult.				
Effector		+		
Recognition	+	+		Cantor and Boyse (1975b)
Delayed-type Hyper-sensitivity	+			Huber et al (1976)

TABLE 1

Suppression of helper responses is seen when Ly23 cells are added to Ly1 cells. This suppression is a function of the number of Ly23 cells added. Cantor et al (1976) suggest that the normal response is a function of a balance between helper and suppressor cells. The fact that cytotoxic cells and suppressor cells are of the same Ly subclass raises the question of whether suppression is mediated by cell killing, but this has not been determined. (Jandinski et al 1976).

Delayed type hypersensitivity (DTH) is measured by foot-pad swelling to SRBC's in irradiated, thymectomized mice reconstituted with bone marrow cells (required for viability) and Ly subclasses of T cells and then sensitized to SRBC's. Huber et al (1976) found that Ly1 cells are required for the induction and expression of the response. Ly23 cells can inihibit both induction and expression of DTH in this system. They suggest that Ly1 cells may be particularly equipped to interact with Ia associated antigen on the surface of macrophages in the generation of antibody (T-B cooperation) and initiation of DTH. Cantor and Boyse suggest that Ly1 cells play an augmenting role in the mixed leukocyte reaction (MLC) by recognition of I region antigens. Cytotoxicity in MLC is performed by Ly 23 cells, but it is amplified by Ly1 cells. The two cell types seem to recognize different antigens coded by regions of the major histocompatibility locus. (Cantor and Boyse, 1975). Although the cells that mediate DTH and those that act as helper cells are both Ly1, they show different responses to tolerogenizing injections of antigen. (Silver and Benacerraf, 1974). This implies that the Ly subclasses are not homogenous.

In addition to the study of T-cell heterogeneity, this system has been used to study the differentiation of precursor cells into effector cells. Huber et al (1976) investigated the question of whether the Ly antigens are two stages in a single line of differentiation (e.g. Ly1 -- Ly23 or Ly23-- Ly1) or products of separate Thymus directed differentiation. They repopulated lethally irradiated thymectomized mice with B cells and then added either Ly1 or Ly23 cells. After up to 20 days afterwards, only the administered Ly subclass was found, and the animals were capable of functions of that subclass and not of those which are known to require cells of the other subclass. This favors one of the following models:



They favor the first model since they have evidence (not yet published) that in some cases Ly123 cells can become Ly23 cells. Indirect evidence that also favors this model is that young mice have a high proportion of Ly123 cells and this decreases with age as the proportion of Ly1 and Ly23 cells rises.

Whichever of these models is correct, something must control the differentiation. One possibility is that exposure to antigen leads to the development of an effector cell from a precursor. Cantor and Boyse (1975) depleted mice of T cells and then restored them with cells of a

particular Ly subclass. They then exposed them to antigen. Mice restored with Ly23 cell could generate cytotoxicity but not helper activity to that antigen. There were no Ly123 cells in the animals, so this suggests that antigen specific Ly23 cells must have existed prior to exposure to the antigen.

One function that has not been studied with the Ly system is lymphokine secretion. As this has been associated with DTH, it might be expected that lymphokine secreting cells will also be Ly1. On the other hand, one of the lymphokines, lymphotoxin (LT), mediates cell killing and this might be an Ly23 cell. However, there is some evidence to suggest that the cell-mediated direct cytotoxicity differs from LT mediated killing. For one thing, cell mediated direct killing is extremely specific, while LT killing is non-specific (i.e. bystander cells are killed). Henney et al (1974) found a difference between their sensitivities to protein synthesis inhibitors, with mediator production being much more sensitive. There was also a difference between their sensitivities to cAMP, which inhibited cell-mediated cytotoxicity, but had no effect on LT production. This evidence does not rule out, however, that these are separate functions of the same cell line, nor that other mediators may be secreted by cells involved in cytotoxicity or suppression.

Tigelaar and Gorczynski (1974) also separated lymphokine secretion from cell mediated cytotoxicity, through the use of a unit gravity sedimentation column. The cells which mediated direct cytotoxicity to an alloantigen sedimented more slowly than the cells which were capable of MIF secretion in response to the same alloantigen. Thus the MIF secreting

cells were larger (see below). They were also able to separate, also less completely, the precursor cells, suggesting that the populations were different before any contact with antigen.

The two functions could also be separated on the basis of differing tissue disbribution and cell migratory properties. Cells mediating specific killing were largely found in the spleen and mesenteric lymph nodes after i.p. exposure to antigen, whereas MIF was produced by cells found there and in peripheral lymph nodes. Sensitized cells showed the same difference in migratory patterns when injected into irradiated mice. None of these methods achieved complete separation between the two functions. (Tigelaar and Gorzynski, 1974).

Tse and Dutton (1976) separated helper activity from suppressor activity on a Ficoll-velocity sedimentation gradient using centrifugation. They used a population of ConA stimulated mouse spleen cells purified on a nylon column. The helper activity, defined by an increase in PFC to sheep RBC's, was found in the lightest fractions, which contained the smallest cells. Suppressor activity, measured by the same assay, was found in the fastest sedimenting fractions which contained larger cells. The suppressor activity was associated with cells which incorporated 3H-thymidine. This suggests that suppressor activity is accompanied by blast transformation, while helper activity is not. Both cells were radioresistant, however.

One problem with this experiment was that the nylon purification removes many of the macrophages. Although 2-ME was added to the final cultures to replace macrophages, it is possible that some of the

separation was due to a change in macrophage concentration and a differential requirement for macrophages. This will be discussed more completely below. Another difficulty is that the experiment studied Con A activated cells, and it is not clear if antigen stimulated cells would show the same sedimentation pattern.

In fact, Gerber and Steinberg (1975) found that unstimulated helper cells were larger than suppressor cells. They used the GVH reaction as an assay system, so it is not clear how this work relates to that of Tse and Dutton. Both of these studies, however, indicate that suppressor and helper cells can be separated both before and after contact with antigen.

In contrast, Durkin et al (1975), studying sensitized rats, found a population of cells which were specifically enriched for soluble antigen responsive cells (as measured by blast transformation, DNA synthesis, and LT production) which were also capable of acting as regulatory cells. They used differences in buoyant density to separate PHA responsive cells from antigen responsive cells, the latter being less dense. They achieved a 4-6 fold enrichment of antigen responsive cells, which were further characterized as being adherent to glass wool. These cells, which were not themselves PHA responsive, were then added to PHA responsive cells. They were shown to either suppress or potentiate the response to PHA partly depending on the number of cells added. The PHA responsive cells were also capable of regulating the antigen response, sometimes enhancing it and sometimes suppressing it. Macrophage addition or killing had no effect in this system. It is not clear what is happening in this system as the factors which determine whether suppression or potentiation will

occur have not been completely characterized. The experiment does suggest that regulatory activity may be a property of cells which also respond to soluble antigen by dividing and secreting lymphokines.

Thus it is clear that T-cells are a heterogeneous population of cells that perform several functions, both effector and regulatory. These cells can be separated on the basis of surface antigens, tissue location, and physical properties. The in vivo immune response is the net result of interactions between T-cells. Within any given response, there is also a change in a population of cells as they encounter and react to antigen to form effector and memory cells. This development has been studied by following populations over time.

Cerottini, MacDonald et al (Cerottini et al, 1974; MacDonald et al, 1974a, 1974b) have studied the cytotoxic reaction in a mixed leukocyte culture (MLC) over time. They could show that the in vitro MLC response was greater when the effector cells came from immune donors, suggesting that there is an anamestic response. In a study of long-term cultures, made possible by the addition of 2-ME which non-specifically increased the viability of those cultures, cytotoxic activity peaked at 4 days and then decreased. Addition of more allogeneic stimulating cells could then restimulate cytotoxic activity. Repeated restimulation led to repeated appearance of cytotoxicity. These secondary responses differed from the primary response in that they occurred earlier and were of greater magnitude. They used the technique of unit gravity sedimentation to separate the cells involved on the basis of size. In the primary response the cytotoxic cells were large cells. If the fractions which gave the most cytotoxicity on day 4 (e.g. the fast sedimenting or large cells) were

cultured without stimulating cells and resedimented at day 14, when little cytotoxicity is present, the population consists mostly of small cells, and it is these small cells which are now capable of generating the secondary cytotoxicity when reexposed to stimulating cells. After reexposure to stimulating cells the population of cells contained many more large cells.

Thus they postulate the following series of events: Small lymphocytes become large effector cells on exposure to alloantigen. This large population then gives rise to a population of small cells which are capable of anamnestic response. On restimulation the small cells differentiate into large effector cells. At the same time there is an increase in cell number. Although without the ability to follow single cells, it cannot be formally proved that the effector cells are the same ones that become small memory cells, they feel that this is the most likely explanation for their results.

One interesting aspect of their findings is that early in the secondary response there is an increase in cytotoxicity without a corresponding increase in cell number. This is then followed by an increase in cell number. This suggests that some of the differentiation from memory to killer cells is independent of cell division. The work of Nedrud et al (1975) supports this as they found that the secondary response in the MLC was resistant to DNA synthesis inhibitors, while the primary response was abolished when DNA synthesis was prevented.

Durkin and Waksman (1974) find an increase in less dense cells (which are generally larger) in lymph nodes recently exposed to soluable antigen. This is followed by an increase in progressively more dense (generally

smaller) cells. This, too, supports a model in which antigen sensitized cells are large, and become progressively smaller with time. They find that the large cells are capable of responding to antigen, however.

III. The role of macrophages

Much work has been done to investigate the interaction of T-cells with other cells, most prominently macrophages. Any study that attempts to separate individual populations of T-cells will also separate them from macrophages, which may lead to the disappearance of a response, not because the T-cells are not capable of that response, but because they need to interact with macrophages.

Macrophages are active in the induction of various types of T-cell mediated immunity, as well as being important effector cells. In this regard, they secrete hydrolytic enzymes, phagocytose and digest debris and foreign antigens. Much work has been done to study the requirement for macrophages in the induction of T-cell activation, whether by mitogens or antigens, both soluble and cell-bound. Unfortunately the work in this field has often been contradictory and confusing.

In order to analyze the work, we must look closely at several factors which may affect the results of any study looking at the need for macrophages in T-cell activation.

(1) Which T-cell function is being studied? The requirement for macrophages may be functionally specific.

(2) What is the composition of the population being studied? Specifically, what is the proportion of T-cells, B-cells and macrophages. Studies claiming to deplete a population of macrophages ought to study pre- and post-depletional cell compositions. Furthermore, as Rosenstreich

and Rosenthal (1976) point out, various techniques of quantifying macrophages may give different results. For example, the technique of latex phagocytosis may not show as many macrophages as esterase staining resulting in the mistaken conclusion that a population has no macrophages when it may have as many as 1%, enough to support some of the T-cell functions studied. Glass adherence is also not completely reliable, as they found that macrophages that are only weakly adherent may still contain significant antigen binding capacity.

(3) What method is used to separate macrophages from other cells and what other cells might be removed by the method? An experiment in which suppressor cells were removed with the macrophages might mistakenly be interpreted as showing an inhibitory effect of macrophages. Some suppressor cells may be glass adherent. (Bach and Waksman, 1973). Additionally, if different populations of T-cells have different requirements for macrophages, separation may result in selection of one population over another. Thus we must be careful not to over-interpret an experiment as saying something about all T-cells, when in fact it investigates only a highly purified population.

(4) What are the conditions of the cultures? Such features as cell density, antigen and mitogen concentration may influence the requirement for macrophages. Thus the cell concentration, shape of culture dish and concentration of stimulants should be noted.

Using a variety of separation systems, investigators have asked two general questions: (1) Is there a requirement for macrophages as accessory cells in T-cell functions, and (2) What roles do they serve? All of the work reviewed was done in vitro, and most used the

proliferative response as an assay system. Less can be said about macrophages and such T-cell responses as suppression or mediator secretion, or about in vivo requirements.

The most confusing results have come from studies looking at the need for macrophages in T-cell activation by PHA. One of the earliest studies is that of Oppenheim et al (1968) who reported that removal of adherent cells from human leukocytes did not change PHA responsiveness at normal PHA doses, but did inhibit the response at suboptimal PHA concentrations. Their purified cell preparations contained 0-2% latex phagocytozing cells after glass bead purification and 0-7% (median 1%) after nylon separation. The decreased response at lower PHA concentration could be restored by an increase in cell density. They suggest that this might allow the remaining macrophages to contact more lymphocytes as well as facilitating T-T interactions if they are important. No attempt was made to add back macrophages. In addition they used latex phagocytosis as their means of counting macrophages which may underestimate the true number of macrophages (see above), so this study is not definitive.

Other investigators have found a similar dose-dependent decrease in DNA synthesis in response to PHA after purification, which can be restored with macrophages. (Alter and Bach, 1970; Folch, Yoshinaga, and Waksman, 1973; Lohrmann et al, 1974). On the other hand, several groups have found an absolute dependence on macrophages.(Greineder and Rosenthal, 1975). Lipsky, Ellner and Rosenthal (1976) passed guinea pig lymph node cells over nylon twice, leaving 0-0.1% macrophages as determined by phagocytosis, esterase staining and glass adherence. Some PHA response remained only at cell concentrations of 10^5/well or greater. This

response was greatly increased by addition of 20-50% macrophages. At lower concentrations of lymphocytes more macrophages were required for optimal responsiveness. Either allogeneic or syngeneic macrophages or fibroblasts were capable of restoring the response, but thymocytes could not. They hypothesize that cell density is an important variable, as a greater number of macrophages were required at lower concentrations. There appeared to be some macrophage independent response at higher cell densities. Varying the dose of PHA seemed to make no difference.

In contrast to these reports, others have found an absolute dependence on macrophages in the activation of T-cells by PHA. Levis and Robbins (1970) studied human leukocytes separated on nylon and then glass, yielding a cell population 99% lymphocytes (morphologically). These purified cells showed only a minimal response to PHA. The response could be restored by as few as 4 x 10^3 glass adherent cells per 10^6 lymphocytes (0.4%). They also noted that the small residual response after purification was greatest in those cultures which later were shown to have deposited some glass adherent cells on coverslips. They suggested that even the small amount of PHA response seen after purification was due to contamination by glass adherent cells.

Rosenstreich et al (1976) also found an absolute dependence on macrophages for a PHA response. They purified guinea pig lymph node cells by passages over two columns: first a glass bead and nylon column and then a column of fine glass beads. Their final population contained fewer than 0.1% macrophages as determined by latex phagocytosis. However, their original lymph node population had also contained fewer than 0.1% macrophages. Using esterase staining they counted 0.3% macrophages in their purified preparation, versus 0.7=0.1% in unpurified cells.

The purified fraction responded only very weakly to PHA. The response was restored to control levels by the addition of 10% macrophages. Soluble factors, secreted by macrophages acting across a double-chambered culture dish, were able to substitute partly for direct contact, providing on the average of 55% of the response seen when the macrophages were in direct contact with the lymphocytes.

These results are somewhat puzzling for it seems to require the addition of 10% macrophages to restore a response to a population that contained 0.8% at most to begin with. It seems likely that more than just macrophages have been removed. The authors are careful to point out that all they can say is that they have isolated a subpopulation of T-cells that require macrophages to respond to PHA. They suggest in fact that they have isolated an especially macrophage dependent population. This would explain why there is so much decrease in response despite so little difference in the number of macrophages.

On the other hand, Waldron et al (1973), who also studied guinea lymph node cells, found that populations purified on acid-cleaned glass beads so that they contained less than 0.2% macrophages (as measured by latex phagocytosis) showed no change in PHA response. Nor was the PHA response influenced by the addition of macrophages (as many as 30% were tried).

Thus it is still not clear whether there is an absolute dependence on macrophages or not. The major difficulty is that it is difficult to completely deplete a population of macrophages, yet, since so few macrophages are capable of greatly enhancing the response, unless the population is functionally devoid of macrophages, no conclusions can be

drawn. Substances that kill macrophages selectively might be useful to study this question. They would also solve the problem that in order to remove macrophages, other adherent cells must also be removed, leaving a population which may not be representative of all T-cells. Nevertheless it is clear that macrophages can play a role in the PHA response, although given high cell densities and optimum concentrations of PHA, some of the response may be macrophage independent. The role of the macrophage will be further reviewed below.

In contrast to the disagreement regarding PHA, there is good agreement that the response to soluble antigen as measured by DNA synthesis, is a macrophage dependent function. This has proved true in such diverse systems as human peripheral blood leukocytes (Alter and Bach, 1970) (Oppenheim et al, 1968), guinea pig lymph node cells (Waldron et al, 1973; Lipsky et al, 1976) and guinea pig peritoneal exudate lymphocytes (Rosenstreich and Rosenthal, 1974).

Seeger and Oppenheim (1970) studying guinea pig lymph node cells found some proliferation in response to antigen after glass bead purification, but significantly more when irradiated macrophages were added. Unpurified lymphocytes also showed an enhanced response when macrophages were added. The maximum response was seen at 20% macrophages. Immune and nonimmune macrophages gave the same response, but the supernatant could not substitute for the cells. They suggest that their response after purification is due to contaminating macrophages. This is supported by the fact that as few as 0.65% macrophages could restore the response.

Rosenstreich et al (1974) point out the difficulty in completely depleting a population of macrophages, but repeated passage over nylon-glass bead adherence columns led to a significant decrease in the antigen induced proliferative response of guinea pig peritoneal exudate cells (which contain a much higher proportion of macrophages than do lymph node cells). The response could be restored with fresh macrophages. In their system, all the residual response could be explained by the 1-2% macrophages the purified population still contained.

Lipsky et al (1976) found that, in contrast to PHA response, only syngeneic macrophages could restore the antigen response.

Several experimenters have found a more strict dependence on macrophages for the response to antigen that the PHA response. Oppenheim et al, 1968; Waldron et al, 1973). The supernatant of macrophage cultures cannot replace actual contact with macrophages. (Seeger and Oppenheim, 1970). In contrast, Rosenstreich et al (1976) found that the number of macrophages required for optimum response to PHA and antigen was the same. No one has tried to see if supraoptimum doses of antigen can replace the macrophages. A small number of macrophages will support the response, but 10-20% seem to be optimum. (Rosenstreich et al, 1976).

Several other T-cell functions have been studied. Greineder and Rosenthal (1975) have shown that the proliferative response generated by the formation of aldehydes on the T-cell membrane is macrophage dependent. As with PHA stimulation, allogeneic macrophages will suffice, although, unlike PHA, fibroblasts will not.

Oppenheim et al (1968) found a decrease in the proliferation in the MLC in human cells after nylon purification, but they do not test

whether it can be restored with macrophages. Alter and Bach (1970) also studied human leukocytes, purified over a rayon-wool column to yield a population with 0.01% macrophages. Purification of both the responder and mithromycin-treated allogeneic stimulating cells abolished the response. However, if either population was unpurified or is allogeneic or syngeneic macrophages were added, the response was restored. The restoration required at least 1% macrophages with a greater response seen with 5 and 10%.

A follow-up report (Bach, 1970) demonstrated that the supernatant of macrophage cultures could replace the macrophages. Similarly, Phillips et al (1972) found that macrophage supernatants could restore MLC reactions to purified populations of responding cells.

Lohrmann et al (1974) isolated a population of human leukocytes which contained fewer than 1% monocytes and was incapable of responding to allogeneic monocyte depleted cells. The addition of 1% syngeneic monocytes partly restored the response, while 10% monocytes completely restored it. The monocyte function was radio resistant. They did not test the supernatants.

Greineder and Rosenthal (1975) found that, when purified guinea pig lymph node lymphocytes were used as responder cells, only allogeneic macrophages could restore the response. These could act as the stimulator cell, as they could act in the absence of allogeneic lymphocytes. Syngeneic macrophages, with or without purified allogeneic lymphocytes, gave little to no response. The macrophages had to be viable, but were radioresistant.

Rode and Gordon (1970) found the need for a supporter cell in the MLC, but did not investigate whether it could also act as a stimulator cell.

Thus the MLC seems to require macrophages, but whether allogeneic or syngeneic cells are needed is not clear. The work of Alter and Bach suggests an indirect role for the macrophages through the secretion of factors, whereas that of Greineder and Rosenthal suggests that the macrophage may itself act as the stimulating cell.

Only a few investigators have dealt with the relationship of macrophages to the secretion of lymphokines. Wahl et al (1975) purified rat lymph node cells on nylon and glass bead columns. Their purified population, which contained $<1\%$ macrophages, could no longer secrete macrophage activating factor or monocyte chemotactic factor. These responses could be restored by the addition of 10%, but not 1%, peritoneal exudate cells. Nelson and Leu (1975) likewise found that 5% macrophages could restore the proliferation and MIF secretion lost when guinea pig lymph node cells were purified on glass beads. Immune or non-immune macrophages gave identical responses. Neither group tested the supernatants for activity.

Yoshinaga and Waksman (1973) studied CFA sensitized rat lymph node cells purified on glass bead columns. Both proliferation and lymphotoxin secretion to response to antigen were abolished by this purification. As few as 0.25% macrophages can restore the proliferative response. Some enhancement is seen with 1%. The lymphotoxin secretion was also restored with macrophages (5% was the only concentration reported).

Wagner et al (1972) studied the cytotoxic response of mouse lymphocytes to cell bound alloantigens and found that it was 50 times decreased after purification over glass beads. It was completely abolished if they used the additional purifying step of treatment with anti-macrophage serum. This response could be restored with 2% macrophages. As few as 0.13% provided some response. The response to subcellular antigens was similarly affected. They did not test the supernatant.

Erb and Feldman (1975) reported that macrophages were required for the generation of mouse T-helper activity. If the antigen was particulate, allogeneic macrophages or 2-mercaptoethanol were effective, whereas soluble antigens required syngeneic macrophages.

Thus macrophages have been found to have a role in all of the T-cell functions studied, but that role may be different for each. Two basic roles have been proposed for macrophages in activating T-cell populations. The first, which requires direct cell-cell contact, is presentation of membrane bound antigen or mitogen to the T-cell or stabilization of antigen or mitogen bound to the T-cell. The second is secretion of soluble factors.

Several lines of evidence have suggested that antigen is bound to macrophages which then interact physically with lymphocytes. If macrophages are exposed to antigen for as little as one hour, washed and added to purified lymphocytes without additional antigen, these lymphocytes transform as well as when exposed to soluble antigen. (Waldron et al, 1973; Rosenstreich and Rosenthal, 1974; Seeger and Oppenheim, 1970). Lymph node cells exposed to 100μg of soluble antigen (PPD) for up to 24

hours and then washed before addition of macrophages were unable to respond, suggesting that they cannot bind antigen effectively without macrophages. (Waldron et al, 1973). (See also Rosenstreich and Rosenthal, 1974). On the other hand although macrophages are required for activation by aldehydes, and perhaps PHA, either the lymphocyte or the macrophage can bind these substances. (Greineder and Rosenthal, 1975). (Rosenstreich et al, 1976). The binding of PPD by macrophages was temperature dependent. Macrophages incubated with PPD at 37°C were much more active in stimulating T-cell proliferation that those incubated at 40C, although there was some activity generated at this temperature. At 37°C the ability to stimulate T-cells increased sharply between 0 and 15, and then increased only slightly by 60 minutes. (Waldron et al, 1974).

All activity generated at 4° was abolished by trypsinization, whereas only 1/2 of that formed after one hour of incubation at 37° was trypsin sensitive. Cells pulsed at 4° for one hour, washed, and then transferred to 37° also developed some trypsin resistance, although not as much as cells kept at 37°. These cells also showed increasing trypsin resistance as they spent more time at 37°.

These experiments suggest that there is a two step process in antigen binding: one which can proceed to some extent at 4° and a second which requires incubation at 37°. This second step renders the antigen resistant to trypsin treatment, but this does not imply that it has necessarily been internalized.

Histological examination of active cultures reveals clumping of cells, suggesting that physical interaction between cells is required. The dependence of some of these functions on cell density, which has been manipulated both by increasing cell number and changing the shape

of the culture dish also argues for the importance of direct cell contact. (Oppenheim et al, 1968; Lipsky et al, 1976).

Lipsky and Rosenthal (1975) showed that macrophages can bind lymph node cells non-specifically (i.e. no antigen is required and no subpopulation of preferentially bound cells could be identified). There were 72 lymphocytes bound per 100 macrophages and 50% of the macrophages bound cells. The binding reaction increased over 60 minutes. Heat-killed macrophages could not bind lymphocytes, nor could those treated with cytocholasin B, vinblastine or cholchicine. They also studied antigen specific immune binding which differed from non-immune binding decreased. The lymphocytes did not have to be capable of cell division to exhibit binding, but if 3H-thymidine is added, it is incorporated only by bound lymphocytes. This binding was found to require syngeneic macrophages. (Lipsky and Rosenthal, 1975b).

Cytocholasin B could decrease macrophage stimulated lymphocyte proliferation to soluble antigen. It acted by inhibiting antigen-dependent binding of immune lymphocytes to macrophages, but did not affect antigen uptake by macrophages. In order to inhibit the T-cell proliferation it had to be present throughout the culture period. A delay of as little as three hours permitted significant DNA synthesis to occur (as measured at 48 hours). (Rosenthal et al, 1975).

These experiments suggest that lymphocyte macrophage interaction may occur in two steps. First, a non-specific reversible binding, and second an antigen sepcific binding which leads to lymphocyte activation and requires syngeneic macrophages. This second step can be inhibited by cytocholasin B. (Rosenthal et al, 1975).

The cell-requiring function of macrophages seems to be (1) binding and "sequestering" of antigen and (2) physical interaction with T-cells in such a way as to allow activation by the bound antigen. In the case of PHA, aldehydes and, perhaps, cell-bound antigen, this direct cell contact may not be needed as the lymphocyte may be capable of binding the antigen on its own.

The requirement for syngeneic macrophages in functions that require direct cell contact is a function of the H-2 locus in mice, such that interaction between T-cells and macrophages is possible if the two share H-2 loci, even if they differ at other genetic loci. Erb and Feldman (1975), in a study using various strains of mice differing only a regions within the H-2 complex, found that T cell-macrophage interaction in generation of T-helper cells required only I-A region identity. Rosenthal and Shevach (1973) found that guinea pig T-cell macrophage interaction required identity at some portion of the major histocompatibility locus.

The second function of macrophages is secretion of soluble factors. No one has studied whether these soluble factors are antigen-specific. Rosenstreich et al (1976), using double-chambered culture dish, showed that macrophages could act at a distance in triggering the T-cell response to PHA. This response was never as great as that seen when there was actual contact between the two cell populations. The greater efficiency of direct cell contact could be due to less loss of factor into the medium, greater efficiency of secretion of factors, or greater efficiency of transformation by PHA when the two cells meet.

Bach and Alter (Bach, 1970) similarly found that macrophages could act through their supernatants in the MLC. Unanue et al (1976) investigated what factors might control the secretion of lymphostimulatory substances by macrophages. Adding substances easily phagocytosed (e.g. antigen-antibody complexes or latex particles) or adding small numbers of activated T-cells both increased the secretion of lymphostimulatory activity. "Activation" of macrophages seemed to decrease such secretion. Thus macrophages may be regulated by the cells they stimulate.

IV. Methods of enrichment

As noted above, all of the studies of T-cells and their functions in cell mediated immunity suffer from the inability to study single cells. To date this problem has not been overcome, but several techniques have been used to try to selectively enrich a population in antigen responsive T-cells. These have included the use of Ly antisera (see above), density centrifugation, selective adherence to antifluorescein columns (Scott, 1976), fluorescence activated cell sorters, selective adherence to antigen-treated macrophages, and unit gravity sedimentation gradients.

Ben-Sasson et al (1975) describe a technique of obtaining specific T-lymphocytes by allowing them to adhere to an antigen-pulsed macrophage layer for 48 hours. However, the adherent population did not show a greater proliferative response than the parent population. Addition of non-immune lymphocytes to both populations led to a significant enrichment in the activity of the adherent population. As they had routinely added macrophages to all cultures, they were unable to explain the requirement for an additional cell. Another difficulty with this method is that many active cells are lost, so that it is relatively inefficient.

It also has the disadvantage that the cells are relatively far along in their differentiation, having already contacted both antigen and macrophages, when they are isolated.

A commonly used method of separation is density centrifugation. This method is capable of separating cells which secrete MIF from others and those which respond to soluble antigen from those which respond to PHA. Pick (1973) found a five fold enrichment of MIF activity in cultures of sensitized cells found in the least dense fractions of his discontinuous gradient. These cells were also the largest. Durkin and Waksman (1975) used this technique to separate OVA responsive rat lymph node cells from PHA responsive cells. They did not achieve a complete separation as there were antigen-responsive cells in all four fractions. At nine days after sensitization, the antigen responsive cells were predominantly in the least dense fraction. Both the cells which incorporated 3H-thymidine and the cells which secreted LT were found in this fraction, whereas the PHA responsive cells were denser. They added macrophages to all the cultures, but this did not change the results. This technique allows the study of cell populations over time, as well as the separation of relatively "virgin" populations.

The technique of unit gravity sedimentation was first described by Richard Miller and R.A. Phillips (1968).

The sedimentation velocity of a spherical cell falling through a fluid under the influence of gravity is given by:

$$S = \frac{2}{9} \frac{(\rho - \rho')}{\eta} g r^2$$

where ρ=cell density, ρ'=fluid density, g=gravity, r=cell radius, and η=coefficient of viscosity of the medium. Although the velocity thus depends on both differences in density and size, Miller and Phillips demonstrate that the actual sedimentation velocity depends mainly on size (e.g. proportional to r^2). They do find some deviation from the separation predicted solely on the basis of size then they separate cells of significantly different densities (erythrocytes with a density of 1.08 vs. lymphocytes 1.06 gm/cm^3), and they suggest density plays a small role in causing the erythrocytes to fall faster than would have been predicted on the basis of their size.

The system uses a very small gradient of very slow density: 1.00^4 gm/cm^3 to 1.009 gm/cm^3. This differs greatly from the average cell density of 1.06, and this also contributes to the fact that density makes only a small contribution in determining the velocity of fall. The gradient is used to prevent mixing of the cells during loading of the column.

The actual velocity will depend on the viscosity of the gradient substance which is in turn a function of its temperature. The velocity is approximately independent of cell shape. Thus in their system

$$S \cong 1/4\, r^2$$

when s is expressed in mm/hr and r in μ.

They suggest that their system, called the STAPUT, has certain advantages over the widely used method of density gradient centrifugation. First, variously differentiated cells do not vary widely in density, whereas they clearly vary in size. For instance they cite a 2-fold difference in cell volume during the cell cycle. Second small changes in

PH or salt concentration can completely change the buoyant density of different cell types leading to difficulties in reproducibility.

The unit gravity sedimentation system has been put to a number of uses in the separation of heterogeneous lymphoid populations. For example, Phillips and Miller (1970) show that they can separate the cell causing the graft-vs-host disease from other spleen or bone marrow cells, and that its sedimentation velocity is the same in both organs. They also isolated a population of cells capable of transferring an immune response to sheep RBC's into irradiated mice. (Miller and Phillips, 1970).

Cerottini et al (1974) (see above) used the system to study the maturation of cytotoxic T-cells over time. Tigelaar and Gorczynski (1974) used it to separate cells which secret MIF in response to tumor antigen from those which were cytotoxic to the tumor. Gerber and Steinberg (1975) separated "suppressor" from "helper" thymocytes active in an GVH reaction.

Several groups have used the unit gravity sedimentation system to study B-lymphocytes (Fidler et al, 1976). Hecht, Ruddle and Ruddle (1976) modified the large gradient used by other investigators to study smaller populations of cells. They used 3 ml of cells (5×10^6/ml), instead of 30 ml, in a 50 ml separation chamber. They found a clear increase in size with increased velocity of sedimentation. They were able to separate small lymphocytes from erythrocytes and larger blast-like cells. They also followed cell differentiation by sedimenting cells at various times after 3H-thymidine labelling and following the fate of the label. They then used fractions enriched for antibody producing cells to fuse with non-antibody producing cell lines in hopes of obtaining a line of specific antibody secreting cells.

Thus unit gravity sedimentation is a powerful tool to study heterogeneous populations of cells, to separate various cell functions one from another, to follow selected cell populations over time, and to obtain cells specifically enriched in cells performing a given function.

V. Delayed-type Hypersensitivity (DTH) in the mouse

In this study the technique of unit gravity sedimentation is used to separate cells sensitized to soluble antigen from other lymph node cells. The response is monitored by incorporation of 3H-thymidine, a marker for cell proliferation. This proliferative response to soluble antigen is an in vitro model of DTH, as it is paralleled by the development of an ear swelling response to the sensitizing antigen (Ruddle, manuscript in preparation).

The mouse has been frequently used as an animal model for DTH (Crowle, 1974), both for in vivo and in vitro studies. Mice can be shown to be capable of developing DTH skin reactions to intradermal injections of antigens to which they had previously been sensitized, either actively or passively (by cell transfer). The development of DTH is dependent, as it is in other species, on the dose and type of antigen and the method (route) of sensitization. (LaGrange et al, 1973)

Several tests have been used to study the in vivo response. The foot pad challenge, skin test reactions, which are technically more difficult, ear swelling, radioisotope incorporation in the ear (Vadas et al, 1975), and systemic reactions have all been used to test the level of response to antigens to which the mice have previously been sensitized. The late (18-48 hr) DTH reaction must be distinguished from the earlier Arthus reactions in skin testing. (Crowle, 1974).

In vitro reactions studied have included blast transformation (measured by DNA synthesis), lymphokine production (MIF, MAF, monocyte chemotactic factor, etc.), interferon production and cytotoxicity both to specific and bystander cells (Ruddle, 1972). These in vitro functions may not always have the same time course as the in vivo reactions (Phillips et al 1972).

Many different antigens have been used to induce DTH, including mycobacterium, many other microorganisms, purified protein (such as OVA), denatured proteins, tissue antigens and sheep RBC's. The antigens are injected in aqueous solution, as whole cells, or in oil-water emulsions (Crowle, 1974).

Many factors affect what sort of an immune response will be generated by exposure to antigen. For example, induction of DTH to purified proteins, such as OVA, requires an adjuvant (either IFA or CFA) (Crowle, 1962). This may be to prevent rapid degradation in vivo as 4-8 days of continued stimulation are necessary for antigens such as OVA to induce DTH. Another function may be to guide the antigen to T-dependent areas in the draining lymph node. These areas may be lipophilic. (Crowle, 1974). Intravenous administration of most antigens will not favor DTH development, perhaps interaction with cells in the spleen leads to an early antibody response which may block DTH induction. (LaGrange et al, 1973). Larger doses of antigen may also lead to more of an antibody response.

The dose of antigen required will be a function of the route of administration as s.q. injection may need more antigen than i.v. sensitization (LaGrange et al, 1973).

Another variable is the ability of the mouse to respond to a given antigen. There is genetic variation between strains in responsiveness to various antigens. The response is controlled by Ir genes which are part of the major histocompatibility locus. (Lonai and McDevitt, 1974; Schwartz and Paul, 1976).

Correlation between in vivo and in vitro phenomenon have been demonstrated in several systems. Phillips et al (1972) found a strong inverse correlation between the degree of DNA incorporation in a unidirectional MLC and the survival of a skin graft from the same strain. A similar inverse correlation was seen between MIF production and survival time. Acceleration of graft rejection was seen after presensitization and this was paralleled by an increase in proliferation in the MLC. The time course of the various reactions tested was not the same. For example, MIF production continued to be present for several weeks after MAF production declined. And MIF production disappeared while strong skin reactions were still obtainable. The proliferative response peaked earlier and disappeared sooner than either MIF or MAF activity.

Ruddle et al (1974) also found a correlation between ear swelling and cytotoxicity to fibroblasts in vitro in mice sensitized with one injection of 120 ug tubercle bacilli in the tail fourteen days prior to testing.

This study, using cells active in a DTH reaction, will use a unit gravity sedimentation gradient to separate specifically sensitized T-cells.

MATERIALS AND METHODS

I. Animals

Six to ten week old BALB/C or C57Bl/6 female mice were obtained from Jackson Laboratory, Bar Harbor, Maine.

II. Sensitization

A one to one emulsion was made of ovalbumin (OVA) in saline and Complete Freund's adjuvant (CFA). The OVA was egg albumin, 5 x crystalline, ICN Pharmaceuticals, Inc. The CFA contained 6 mg/ml heat killed tubercle bacilli D, Pt and N strains (Central Veterinary Laboratories, Weybridge, England) in 85% Bayol F, 15% Arlacel A (Esso Refining Company, Bayonne, NewJersey). Each mouse was injected subcutaneously in the tail with 0.04 ml of the emulsion. The total dose of OVA was 100 µg.

III. Lymph node preparation

Eight days after sensitization the animals were sacrificed with ether and the draining inguinal lymph nodes were removed and put into Hanks Balanced Salt Solution (HBSS) (Grand Island Biological Company, Grand Island, N.Y.). They were teased apart, the cells centrifuged at 1000 rpm for 10 minutes and resuspended in medium. The medium consisted of RPMI-1640 with 25mM HEPES buffer, and glutamine supplemented with 5% fetal calf serum (FCS) which had been heat inactivated by incubation in a 56°C water bath for 30 minutes, and antibiotics, 100U/ml penecillin and 100mcg/ml streptomycin. All were obtained from Grand Island Biological Company. The cells were counted with a hemocytometer or a Coulter Counter (with settings of amplification=1, aperture current=$\frac{1}{2}$, threshold=15) and diluted

to the desired concentration. Normal cells were obtained by removing the peripheral lymph nodes of unsensitized mice. They were then treated like the sensitized cells.

IV. Cultures

Macrocultures: The lymph node cells were grown in 1 ml medium in loosely capped tubes (Falcon plastics) with OVA or mitogen added in 0.1 ml medium.

Microcultures: The cells were grown in V bottomed microtiter plates (IS-MVC-96; Linbro, Hamden, Ct) in 0.1 ml medium with OVA added in 25 or 50 µl. The OVA was added with either a 25 µl dropping tip or a Pipetman.

All cultures were incubated at 37 C in a humidified atmosphere of 95% air and 5% CO_2.

V. DNA synthesis

After 54 hours in culture 1µCi 3H-methyl thymidine (specific activity 1.9Ci/mM, New England Nuclear). Eighteen hours later the cultures were harvested onto Millipore filters with a Millipore sampling manifold. Each culture tube or microtiter well was washed twice with saline. The saline was added to the manifold. The DNA was precipitated with 5% cold trichloroacetic acid for 20 minutes. The filters were dried and put into 10 ml of Econofluor (New England Nuclear). The radioactivity was determined with a Beckman scintillation counter. All results are expressed as the mean cpm of duplicate or triplicate cultures the standard error of the mean. The stimulation index, S.I. is defined as:

$$= \frac{\text{mean cpm in the presence of antigen}}{\text{mean cpm in the absence of antigen}}$$

VI. Nylon purification

In some experiments the cells were purified by passage over a nylon column by a modification of the method developed by Julius et al (1973) Nylon (Fenwal Leukopak, Fenwal Laboratories, Morton Grove, Ill) was boiled in .2NHCl for 30 minutes, washed with glass-distilled water for 5 days, with frequent changes of water, and then dried at 37° for 3 days. 600 mg of dried nylon was packed into 6 cc in a plastic syringe and autoclaved.

The syringe was saturated with RPMI-1640 supplemented with 10% heat inactivated FCS and allowed to equilibrate at 37° C for one hour. 5-8 $x10^7$ cells in 1 ml medium were added to the column which was incubated for 45 minutes at 37° C. The effluent was harvested by dropping medium onto the column until 25 ml had been collected from the bottom.

VII. Macrophages

Unsensitized mice were injected i.p. with 1 ml sterile 10% proteose peptone. Three days later peritoneal exudate cells were harvested by flushing the peritoneal cavity with 3 ml HBSS. The cells were then spun and resuspended in medium and counted with a hemocytometer. (Erb and Feldman, 1975)

VIII. Unit gravity sedimentation gradient

This gradient was developed by Hecht, Ruddle and Ruddle (1976) as a modification of one described by Miller and Phillips (1968). The cells were suspended in 3 ml of 0.5% bovine serum albumin (BSA, Calbiochem) made up in PBS, at a concentration of 5-8 x 10^6 cells/ml.

The cells were put into a 50 ml sterile plastic syringe (the separation chamber) on top of three sterile glass beads, used to prevent mixing of the cells and medium during gradient formation. The 50 ml continuous 1-3% BSA gradient was prepared by a two chanbered LKB gradient-making device containing 25 ml each of 1% and 3% BSA and pumped into the separation chamber underneath the cells by means of an LKB ReCychrom peristaltic pump. The BSA displaced PBS, which had been previously pumped into the connecting tubing, into a reservoir connected between the pump and separation chamber. Both the reservoir and the separation chamber had three way valves to direct the flow of liquid. After the cells were pumped up the gradient, they were allowed to sediment at room temperature for 4 hours. 2.5 or 5 ml fractions were collected by drops out a 20 guage needle connected to the bottom of the separation chamber.

For microscopic analysis, 0.2-0.5 ml of each fraction were put into a cytocentrifuge (Shandon Cytocentrifuge) and spun onto slides at 1000 rpm for 10 minutes. The slides were fixed in methanol for 10 minutes and stained with 10% Giemsa for 10-15 minutes.

IX. Mean Cell diameter

The mean cell diameter of each fraction was calculated from plots made of size-distribution made by the Coulter Counter plotter. Calibration was done with ploystyrene beads.

X. Mitogens

PHA and LPS were obtained from Difco. The LPS used was 50 µg/ml and the PHA 18 µg/ml, both added to 1 ml cultures in 0.1 ml medium.

RESULTS

1. The assay

The experiments in this study involved assaying for an increase in DNA synthesis using stimulation of 3H-thymidine incorporation. The advantages of this method are its technical simplicity and the small number of cells required. Furthermore, it has been widely used by other investigators, so much is known about the system. There are a few disadvantages. The assay is relatively non-specific, with a varying degree of backround activity. The interpretation of a given amount of incorporation is not entirely clear, as it could be due to rapid cell division on the part of a few cells or slower cell division on the part of many. (Ruddle, 1972) Nevertheless, it provides an easy and clear way to study DTH in vitro and to monitor the antigen responsiveness of a population of cells.

2. Sensitization

The mice received a sensitizing dose of 100µg OVA subcutaneously in the tail in complete Freund's adjuvant. This method of immunization leads to an in vivo response with the characteristics of DTH (Ruddle, manuscript in preparation). The proliferative response was tested in Balb/c cells harvested 5, 8, 12, and 15 days after sensitization. Both the yield of cells per lymph node and the S.I. were maximum at eight days after sensitization. At five days after sensitization, there was some increase in 3H-thymidine incorporation as compared to control cells, but the background incorporation (e.g. sensitized cells grown in the absence of antigen) was also higher. By 12 and 15 days after sensitization, the response had decreased.

This is similar to the time course seen by others. (Durkin and Waksman, 1975; Vadas et al, 1975) In subsequent experiments, cells were harvested eight days after sensitization.

3. Growing small numbers of cells in culture.

In order to study a small number of gradient separated cells, a method of grwoing a small number of cells in culture had to be used. Levinson et al (1974) reported that they could get a response to PHA with 5×10^5 human cells in 1 ml medium and as few as 5000 cells in 0.1 ml medium in microtiter plates. I attempted to duplicate this using OVA as the stimulant. Figure 1 shows sensitized cells in 1 ml medium ("macrocultures"). 2500µg of OVA was added to some tubes in 0.1 ml medium. Significant stimulation was seen with 5×10^5 cells, but not with fewer. The S.I. in this experiment was 2.3. In other experiments at this antigen concentration similar S.I.'s were obtained. Normal cells, obtained from the peripheral lymph nodes of unsensitized mice, showed no stimulation in response to antigen (Figure 1). Their background values were very similar to those of sensitized cells without added antigen.

Figure 2 shows the results of several "microculture" experiments. At 5000 cells/well a small stimulation was sometimes seen, but it wasn't until 50,000 (5×10^5/ml) cells were used that good stimulation was relibly achieved. Table 2 shows the results of several different experiments. One cannot compare one experiment to another as the background values vary, but in general, at 50,000 cells/well, a stimulation index of 3-4 was seen. Again, unsensitized cells showed no response to added antigen. In the separation exper-

iments, 5×10^4 cells/ well were used.

4. Antigen dose

In the original experiments done with lyphocytes in culture, $1\text{-}2 \times 10^6$ cells/1 ml are used. The usual antigen dose has been 25-100µg/ml, with 100µg considered a "large" dose (Waldron et al, 1973). In these experiments we used a much smaller cell number, 5×10^4, and have found that they did not respond to 2.5 or 25µg/ culture (25-250µg/ml) in most cases. Therefore we have used 250µg/ culture (2500µg/ml) as our usual dose. When 5×10^5 cells in 1 ml were used, as little as 25µg of OVA gave a good response. It is not clear why the small number of cells requires so large a dose of antigen for stimulation.

5. Nylon separation

Several observations suggested that this was a T-cell response. It was associated with an ear swelling response, abolished by anti-theta antiserum, and it was associated with the production of lymphotoxin (Ruddle, manuscript in preparation). However, we felt that passage over nylon might increase the purity of the separated population, as well as ensuring that there was no B-cell contribution to the response. Nylon purification was developed by Julius et al (1973) for the purpose of removing B-cells. In addition to removing B-cells, it also removes some of the macrophages.

We put $6\text{-}8 \times 10^7$ cells onto a nylon column and incubated it at 37° for one hour. The cells hwere harvested by washing warm medium through the column until 25ml had been collected. Using this technique, I recovered 25-50% of the cells.

Response to stimulation by PHA and LPS were used to test for selective removal of B-cells. The results of one experiment, done with unsensitized cells, is presented in Table 3. Most of the LPS stimulated activity is removed by passage over nylon, while the PHA stimulated activity remains. Sensitized cells retain their ability to respond to antigen after nylon passage (Table 3).

6. Unit gravity sedimentation

For separation at unit gravity, cells were put in 3 ml of 0.5% bovine serum albumin (BSA) at a concentration of 5-8 x 10^6/ml (for a total of 1.5-2.4 x 10^7 cells per column). They were placed in the neck of a 50 ml plastic syringe and pumped to the top by a continuous gradient of 1-3% BSA. All this was done under sterile conditions. The gradient maker was sterilized under UV light and then washed through with sterile PBS before use. All other permanent parts were autoclaved. This microsystem was developed by Hecht, Ruddle, and Ruddle (1976).

The cells were allowed to sediment at room temperature for four hours, and then harvested by drops from the bottom. Most investigators have used the unit gravity sedimentation system at 4°C, so our sedimentation velocities are faster per cell size, but as we used a constant temperature for all columns, the viscosity of the gradient was constant and the sedimentation velocity is still a function of cell size: $s = kr^2$.

We harvested the cells into ten 5 ml fractions. In later experiments, we split fraction 7 into two halves: 7a and 7b. Fraction 1 contained the fastest sedimenting cells. Due to the small

numbers of cells in the fastest falling fractions, fractions 1,2, and 3 were pooled, as were fractions 4 and 5, for the purpose of growing the cells.

After collection from the column, part of each fraction was used to make slides using a cytocentrifuge. The rest was centrifuged and resuspended in medium. The cells were counted using either a hemocytometer or a Coulter Counter and adjusted to 5 x 10^4 cells/0.1ml for microculture.

The total yield after harvesting and spinning the cells varied from 40-95%. Fraction 8 always contained the most cells (usually more than 25% of the total cells loaded on the column). Fractions 9 and 7 (or 7b when 7 was split) also contained a sizable proportion of the cells. Fractions 1-6 contained less than 10% of the cells, and most always less than 5%. Some typical yields are graphed in Figure 3.

A size vs. frquency plot was made of each fraction on a Coulter Counter plotter. One experiment is shown in Figure 4 (a-h). From this the mean cell volume of each fraction was calculated on the assumption that the cells were spherical. Calibration was done with polystyrene beads. The results are shown in Table 4. The largest cells are contained in fractions 4-7a. The rest of the fractions, including fractions 1-3 contain cells with smaller mean cell volumes. Fractions 9-10 and 1-3 contain two peaks of cells. It is possible that fractions 1-3 contained some cells that are too large to be plotted on this scale, but there are so few cells in these fractions that they were not investigated further. The separation,

as determined by the plots, was certainly not complete, and in some other experiments the mean cell volumes of the fractions 4-7a were not so clearly separated from those of the unseparated population, although they always contained a greater proportion of larger cells. The actual sedimentation velocity of each fraction is also shown.

Cells obtained from individual fractions were grown in culture with or without added OVA. After 54 hours in culture, 0.1μci of 3H-thymidine was added to each well. The cells were harvested 18 hours later (for a total of 72 hours in culture) as described above. Results of several separations are shown in Figures 5-7. It is clear that the majority of cells, contained in fractions 8 and 9 showed no response to added antigen, whereas fractions 4-6 and to a lesser degree fraction 7 showed stimulation indices of greater than the unseparated cells. In fact in one case the controls did not show any increased incorporation in response to antigen, despite the fact that antigen-specific activity was clearly recoverable in fractions 4-6.

The background counts in culutres without antigen were very low in the early experiments done with BALB/c cells, but in the later experiments it was much higher, especially in the fastest sedimenting fractions. (Figure 8) Whether this is a function of the fact that C57Bl/6 mice were used or due to a different batch of fetal calf serum is not clear.

The best separation was seen in the experiment graphed in Figure 6, where fraction 6 showed 3591 cpm in the presence of antigen vs 262 cpm for the unseparated cells. Although in this exper-

iment there were not enough cells in fraction 6 to run controls (i.e. no antigen added), other experiments run at this same time demonstrated that fraction 6 never showed more than 300 cpm without antigen (Figure 8).

These fractions showed a significant enrichment for antigen responsive cells. As they contain only a small percentage of the cells, the major population of unresponsive cells can be separated from a minor population of cells which have an enhanced responsiveness to antigen. These cells can be triggered to proliferate after reexposure to specific antigen, but do not proliferate unttil encountering the antigen.

It is not possible to calculate exactly the extent of the enrichment, as the 3H-thymidine incorporation cannot be directly related to the number of cells dividing. Since the cells are not active before exposure to antigen, there is no morphological way of counting responsive cells. As cytotoxicity to bystander cells is proportional to the number of active cells, this technique would allow the quantification of the enrichment. This is currently being studied (Ruddle, manuscript in preparation).

Two other experiments are shown with similar results. The lack of activity in fraction 5 in Figure 5 is unexplained. In Figure 7 the background is higher, but fraction 6 still shows an increased S.I. and the highest incorporation. Again, the activity is contained in a small percentage of the cells.

Differentials were done on slides made from each fraction of the gradient. These were done without knowing which fraction they

were. Small lymphocytes were cells with minimal cytoplasm. Medium and large lymphocytes contained more cytoplasm. Macrophages were defined as large cells with lighter staining cytoplasm and nuclei. Their cytoplasm seemed to be less dense. The results of a differential are shown in Table 5.

Fractions 1-3 contain very few cells, including some clumps of cells and much debris. Fractions 4-6 contain larger cells and most of the macrophages. Fractions 7b and 8 have smaller cells and fewer macrophages. Fraction 9 contains a uniform population of small darkly staining cells, as well as some RBC's. Fraction 10 contains a mixture of cell types. Most of the cells are small lymphocytes, but not all small lymphocytes have the same diameter. Thus the average size of cells scored as small lymphocytes may well differ between fractions, as suggested by the difference in mean cell volumes seen when the sizes were plotted on the Coulter counter.

Unit gravity sedimentation gradients were also run with nylon purified cells. The yields in each fraction were very similar to those obtained without nylon purification, with fraction 8 still containing the greatest number of cells (Figure 9).

Two separations are presented in Figures 10 and 11. These results are very similar to those obtained without nylon purification, with fractions 4-7a containing almost all of the antigen-specific activity despite the fact that they contain only a small minority of the cells. In one experiment (Figure 11), no activity was seen in the control cells (unseparated) either before or after nylon purification. But fraction 6 showed a peak incorporation of

2719 cpm (vs 668 for the cells without antigen). In one experiment (figure 10), fraction 4 contained the highest cpm, with fractions 6 and 7a also having S.I.'s greater than those obtained with unseparated cells.

I have therefore been able to separate a smallproportion of specifically activatable cells from the majority of lymph node cells. Nylon purification, which removes B-cells, does not affect the separation in any major way. Thus nylon passage does not abolish the activity, nor does it seem to enrich for it.

Examination of the slides prepared from nylon purified, column separated cells revealed that the faster sedimenting fractions again contained a larger proportion of medium and large lyphocytes. Two experiments are shown in Tables 6 and 7. In one case no cells with the morphology of macrophages were seen, while in the other as many as 4% (fraction 6) were counted. The reason for this difference is not known, although a similar phenomenon was noted by Oppenheim (1968) who found that there were 0-7% macrophages (median 1%) after separation of human blood leukocytes on nylon.

7. The role of macrophages

Although it seems that we have been able to separate an antigen responsive population of larger cells from an inactive population of smaller cells, one other possible explanation is that the active population contains the macrophages and that these account for the increase in activity. If this were the case, fraction 8 might contain potentially antigen-responsive cells which could be triggered if there were macrophages present. This is of concern for several

reasons. (1) It is known that antigen-triggered proliferation requires macrophages, and that only a few macrophages can make a great difference in the level of activity seen (see introduction). Lymph nodes normally contain very few macrophages, especially after nylon purification, and a small enrichment might greatly increase the proliferation. (2) Macrophages are larger cells than lymphocytes and thus will be found in the faster sedimenting fractions, where we also see the antigen specific activity. (3) Counts of the fractions have suggested that the macrophages are found in the same fractions that we see the activity. However, counting macrophages is not a good way of measuring them as there are so few that counts are not very accurate. In addition there may be functional macrophages that do not have the typical morphology.

To analyze the effect of macrophages directly, we added 5% peritoneal exudate cells to each fraction. The results are presented in Figures 7 and 12. In this experiment, 2.5×10^3 peritoneal exudate cells, obtained from unsensitized mice injected three days earlier with 1 ml of 10% proteose peptone i.p. Erb and Feldman (1975) present evidence that cells obtained this way are mostly macrophages and can be used without further treatment. Figure 7 shows the results without added macrophages and Figure 12 shows the results when macrophages are added. The macrophages themselves showed no incorporation (data not presented). In both cases fraction 6 shows the highest incorporation. Addition of the macrophages did not restore any antigen specific activity to fraction 8, although it seemed to non-specifically increase incorporation of 3H-thymidine

in all the slower sedimenting fractions. The cpm in fraction 6 was decreased, but it still showed enrichment as compared to the controls.

It is risky to overinterpret these data, as they represent only one experiment, but the results suggest that the absence of antigen-specific DNA synthesis in the majority of cells, found in fraction 8 after sedimentation, is not due to the absence of macrophages. The non-specific increase in 3H-thymidine incorporation may be due to a general increase in cell viability in the presence of macrophages.

Another way to investigate this question would be to add cells from fraction 6 obtained by separation of normal lymph node cells to those of fraction 8 derived from sensitized cells. If the lack of activity in fraction 8 is due to the absence of macrophages or a non-specific helper cell, the cells from a normal fraction 6 ought to act synergistically with those of fraction 8 to restore activity.

DISCUSSION

The experiments reported here have demonstrated that a highly active population of antigen-responsive cells, which sediment at a faster rate than the majority of lymph node cells, can be separated from the non-responding, slower sedimenting cells using the technique of unit gravity sedimentation. We have been able to characterize the various fractions with techniques of tissue culture that use a small number of cells. The activity is resistent to one passage over nylon and does not seem to be specifically affected by the addition of 5% macrophages.

The cells that we isolate are T-cells active in a DTH reaction in vivo (Ruddle, manuscript in preparation) and are specifically sensitized to a soluble protein antigen, OVA. This system is a model for this one type of T-cell function.

Although Levinson et al (1974) had reported that as few as 5000 human cells could respond to PHA in culture, I was only able to get a consistent response with 5×10^4 cells per microtiter plate well. With this number of unseparated cells, I found an incorporation of 3H-thymidine of about 800 cpm in response to OVA, representing a S.I. of 2.5-4.

During the time that this work was in progress, Schwartz and Paul (1975) described an assay they had developed for T-cell function in which they harvested peritoneal exudate cells from mice 1-2 weeks after hind foot pad immunization with 5-20 µg of antigen. The peritoneal cells were harvested five days after i.p. injection of 1 ml of 10% Brewer's thioglycollate and purified on a nylon column before use. The cells were cultured at 1×10^4 to 3×10^5 cells/

well in a round bottom microculture plate with an antigen concentration of 0.05 to 500µg/ml. Their final nylon purified population contained 5-8% macrophages, 45-80% lymphocytes, 1-4% B-cells and 25-50% granulocytes (variations depending on the strain of mouse used). The yield of lymphocytes was only 3-5 x 10^6 per mouse. With this system, at an antigen concentration of 50µg/ml, they saw good stimulation with as few as 1.25 x 10^4 cells (in 0.2ml). They got about 1600 cpm which increased sharply to greater than 40,000 cpm at 2 x 10^5 cells/well (control of about 3000 cpm) representing a stimulation index of about 13. With as little as 0.05µg/ml of antigen (0.01 µg/culture), 2 x 10^5 cells gave some stimulation. It is not clear exactly how this system relates to the one described above, as they use a completely different population of cells and harvest at a different time after sensitization. Our system has the advantage of higher yields (meaning that fewer mice are required) and less contamination with other cells, such as granulocytes and macrophages.

Factors which affect the level of proliferation seen in culture have not been totally worked out, as Lonai and McDevitt (1974), using peritonal exudate cells obtained in a similar, although not identical manner, found much smaller cpms with many more cells. Their method differed in detail (different strain of mice, different peritoneal irritant, etc), but the striking difference in the extent of antigen stimulated DNA synthesis is unexplained, pointing out the fact that there is much that we do not know about what controls the response in these cultures.

Some of the factors which may affect the level of response are: (1) the cell population used, (2) the number of cells in culture, (3) the shape of the culture dish, and (4) the concentration of antigen.

Rosenstreich et al (1973) have shown that sensitixed cells obtained from the peritonal cavity after non-specific irritation have a higher activity, as measured by proliferation and MIF production than peripheral lymph nodes. They conclude that this higher activity is only partially explained by the higher numbers of macrophages. It may be due to the preferential migration of activated cells into the irritated peritoneal cavity.

In the system reported here, there is an increase in 3H-thymidine incorporation with increasing numbers of cells (Figure 1). Schwartz and Paul (1975) found a similar increase. In some cases there was inhibition at very high cell densities. Despite the fact that more cells give a greater stimulation, a small number of cells can be used to monitor the response to antigen. This is important as the fractions we separate contain only a small number of cells.

We used conical culture dishes for growing small numbers of cells to optimize cell-cell interactions. This technique has been shown to allow increased proliferation in glass purified cells. (Oppenheim et al, 1968). I could obtain antigen induced proliferation with 1/10 the number of cells in conical microtiter dishes as I could in larger tubes. The larger tubes contained fewer cells/ml of medium, but since the cells fall to the bottom, it is not clear how great a contribution the volume of the medium plays

in determining the response.

In these microcultures, a large quantity of antigen was necessary to obtain stimulation. Thus although 25 µg OVA can stimulate 5×10^5 cells in 1 ml medium, 250 µg was needed for the smaller cultures. Schwartz and Paul (1975) and Lonai and McDevitt (1974) found that stimulation icnreased with the dose of antigen used. In most cases the highest response was seen with the highest dose tested, although PPD stimulation peaked at a rather low dose in Schwartz and Paul's study. I am not aware of anyone who has routinely used as high a dose as I have. There may be an inverse relationship between the number of cells grown and the amount of antigen required for good stimulation. The reason for this is not known.

Unit gravity sedimentation is a method of cell separation developed by Miller and Phillips (1968). It separates cells mainly on the basis of size, although density plays a small role in determining the velocity of sedimentation and may affect the separation when two cells differ greatly in density. This method has been used to study many lymphoid populations. It can separate suppressor from helper cells (Gerber and Steinberg, 1975), isolate cells responsible for cytotoxicity in the MLC (Cerotinni et al, 1974), and study the differentiation of populations over time (Hecht et al, 1976).

Our system was modified from that of Miller and Phillips by Hecht et al (1976). This system uses only 2×10^7 cells and can be run sterilely. The gradient maker is sterilized under uv light

and then rinsed with sterile PBS. The column itself is a sterile, disposable 50 ml syringe. All other parts are autoclaved.

This method has several advantages over other methods of isolating antigen responsive cells. As compared to density centrifugation, it is based on cell size rather than density. Cell size is known to change dramatically with changes in cell cycle and differentiation (Miller and Phillips, 1974). Our gradient uses only a small amount of protein compared to the density centrifugation gradients, an advantage because of the possibility of non-specific stimulation by protein factors in tha BSA. Finally cell recovery may be as great as 95%.

We have shown that we can reliably isolate a population of larger cells in fractions 4-7a which are enriched in antige-responsive cells. These cells sediment at 7.875-15.75 mm/hr. This is faster than other lymphoid populations isolated with this method (e.g. Cerotinni et al, 1974), but this is most likely due to the fact that we let the cells sediment at room temperature instead of 4 C. The separation is not complete as there are mall cells in each fraction, but Coulter plots reveal that the fractions differ in mean cel volumes. Density may play some role in the separation so that some of the small cells in the rapidly sedimenting fractions may be significantly denser than small cells sedimenting more slowly. Another possibility which couls contribute to the rapid sedimentation of some small cells may be clumping, with the cells falling as a unit at a velocity determined by the radius of the unit. Except for some clumps in fractions 1-3, this is unlikely to be the reason, as

nylon purification, which seems to decrease clumping, does not change the yield/fraction or eliminate the small cells in the lower fractions.

Fractions 4-7a, which contain most of the antigen-specific activity, contain larger cells and macrophages. Fractions 7b and 8 may contain some antigen responsive cells, but in such a small proportion that they do not show any stimulation at the cell concentrations used in this study. Fraction 9 contains a population of uniformly small darkly staining lymphocytes and shows no antigen response.

The same fractions which show antigen-stimulated proliferation also produce lymphokine (Ruddle, personal communication). This suggests that the same population of cells can respond to antigen by proliferation and lymphokine secretion, but whether the same cell is capable of both dividing and secreting lymphotoxin is not known.

The activity persists after nylon wool purification. Nylon was first used by Julius et al (1973) to remove B-cells from spleen and lymph node populations, thus enriching for T-cells. Although some T-cells are also removed by the nylon, the passed populations have usually been found to be capable of T-cell functions such as cytotoxicity and helper activity. Greaves and Brown(1974), using a similar technique, found that their nylon passed cells contained less than 3% B-cells and less than 0.05% macrophages (as determined by morphology). Erb and Feldman (1975) found a loss of helper activity after nylon purification which they attributed to a loss of macrophages. On the other hand, if a population contains a large

proportion of macrophages to begin with, nylon will not remove them all. Schwartz and Paul (1975) found that their peritoneal exudate cells contained 5% macrophages after passage over nylon. In some of our slides of nylon-purified cells there are macrophages (Table 8) Furthermore, morphology alone may not reliably identify all the macrophages as there are cells with antigen binding capacity which do not have the typical morphology of macrophages. Thus the activity remaining after nylon purification is a function of T-cells, not B-cells, but cannot be said to be macrophage independent.

The macrophages seem to fall with the larger lymphocytes and are found in the faster sedimenting fractions where the antigen stimulated proliferation is also seen. Nevrtheless, several lines of evidence suggest that we have achieved a true separation of activatable T-cells and not just of macrophages. First, the size of the lymphocytes increases with sedimentation velocity. Second, several other investigators have been able to separate activated T-cells using assay systems which do not require added macrophages (Gerber and Steinberg, 1975, who used an in vivo assay system to monitor separation of suppressor from helper T-cells). As a direct test, we added 5% macrophages to each well and did not find restoration of specific activity in fraction 8 (as would have been expected if it contained a sufficient number of sensitized cells).

Thus the separation is real. The macrophages probably co-sediment with the larger activated cells.

The strength of this system is that a large proportion of cells which do not respond to antigen (in fractions 7b-10) can be separ-

ated from a minor population of active cells, thus greatly enriching for specific cells. These cells have been isolated at an early stage in their differentiation. This is an advantage as compared to other methods (such as that of Ben-Sasson et al, 1976) which require adherence of cells to antigen treated macrophages, or methods which require alteration of the cell membrane (Scott, 1976).

The population we isolate cen thus be used in one of several ways: (1) to assay for antigen responsiveness by stimulation of DNA synthesis or LT secretion, (2) to study membrane properties or antigen binding and triggering of a population known to be greatly enriched in antige-responsive cells. Forexample, the Ly surface antigens of these cells can be studied. Although DTH has been shown in a slightly different system to be a function of Lyl cells, a pure population of cells will allow verification of this as well as the study of the Ly antigen found on cells secreting lymphtoxin. (3) The cells can be fused with established cell lines in an attempt to develop an antigen specific T-cell line. For this last experiment it is essential to have a population as pure as possible to increase the chance of fusion with an antigen-sensitive cell. Work is in progress to fuse cells from fractions 4-7a with a T-cell lymphoma.

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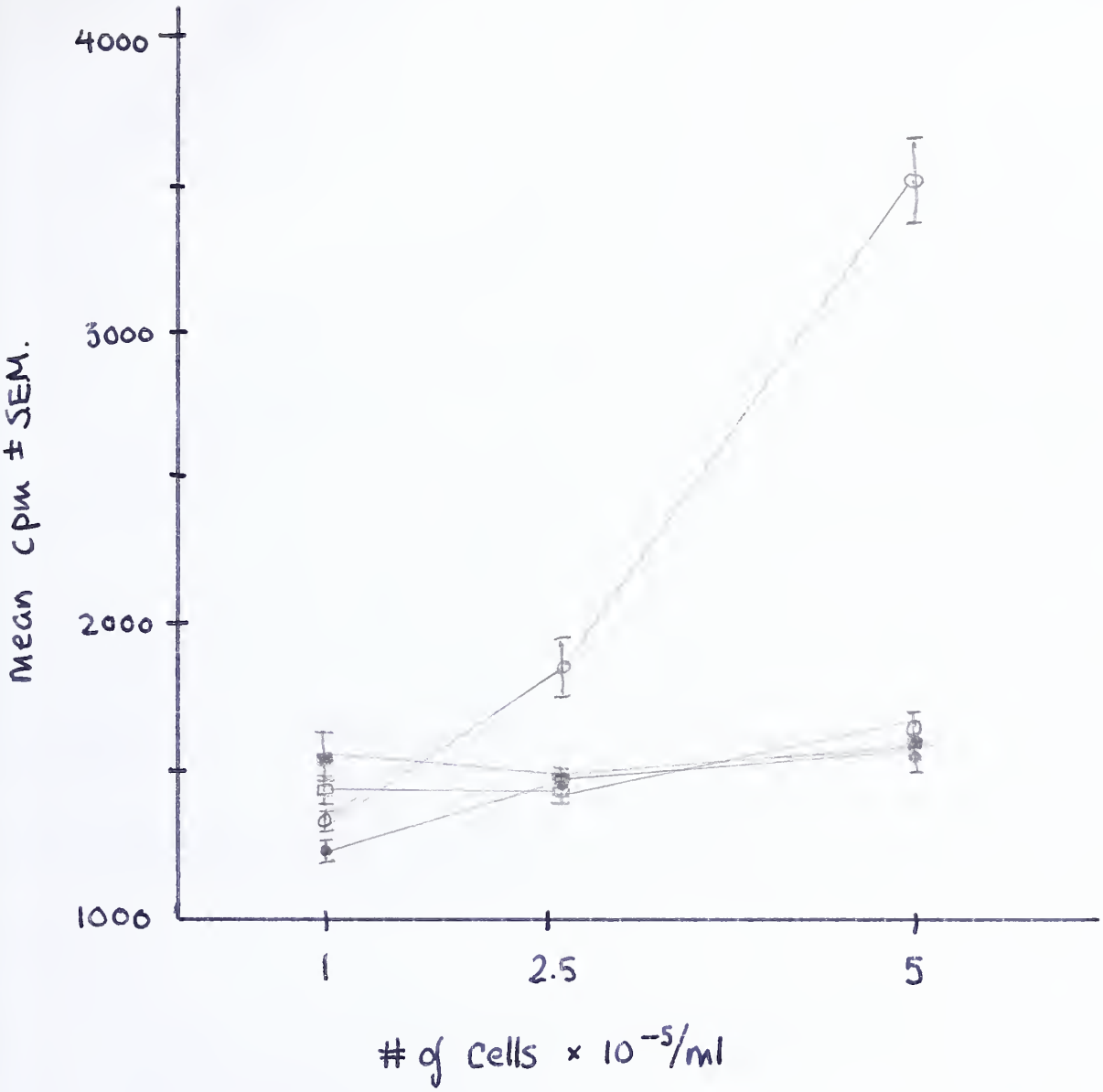


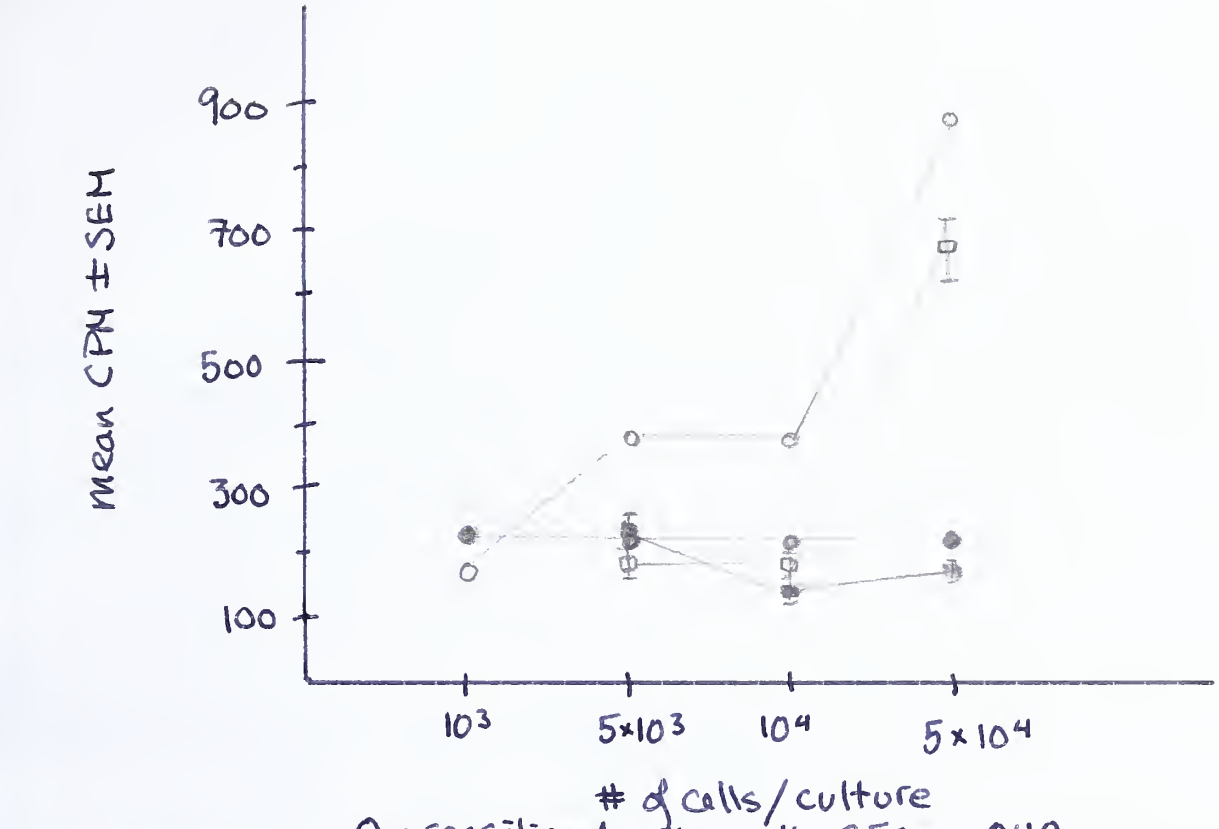
The relation between # of cells and response to antigen in macrocultures (1ml). All cells were obtained from BALB/C mice.

Figure 1

total cells	mean cpm with 250ug OVA	mean cpm without OVA	S.I.
10^3	183.9	236.3	.78
5×10^3	623.4	545.0	1.1
	375.7	203.6	1.8
	196.3	203.5	.85
10^4	195.6	146.7	1.33
	369.3	214.7	1.7
5×10^4	825.6	205.5	4.0
	664.3	170.8	3.9
	495.4	149.3	3.1

Microcultures of cells obtained from sensitized BALB/C mice.

TABLE 2



experiment #7
- ○ - sensitized cells with 250 µg OVA
- ● - sensitized cells without OVA

experiment #8
- □ - sensitized cells with 250 µg OVA
- ■ sensitized cells without OVA

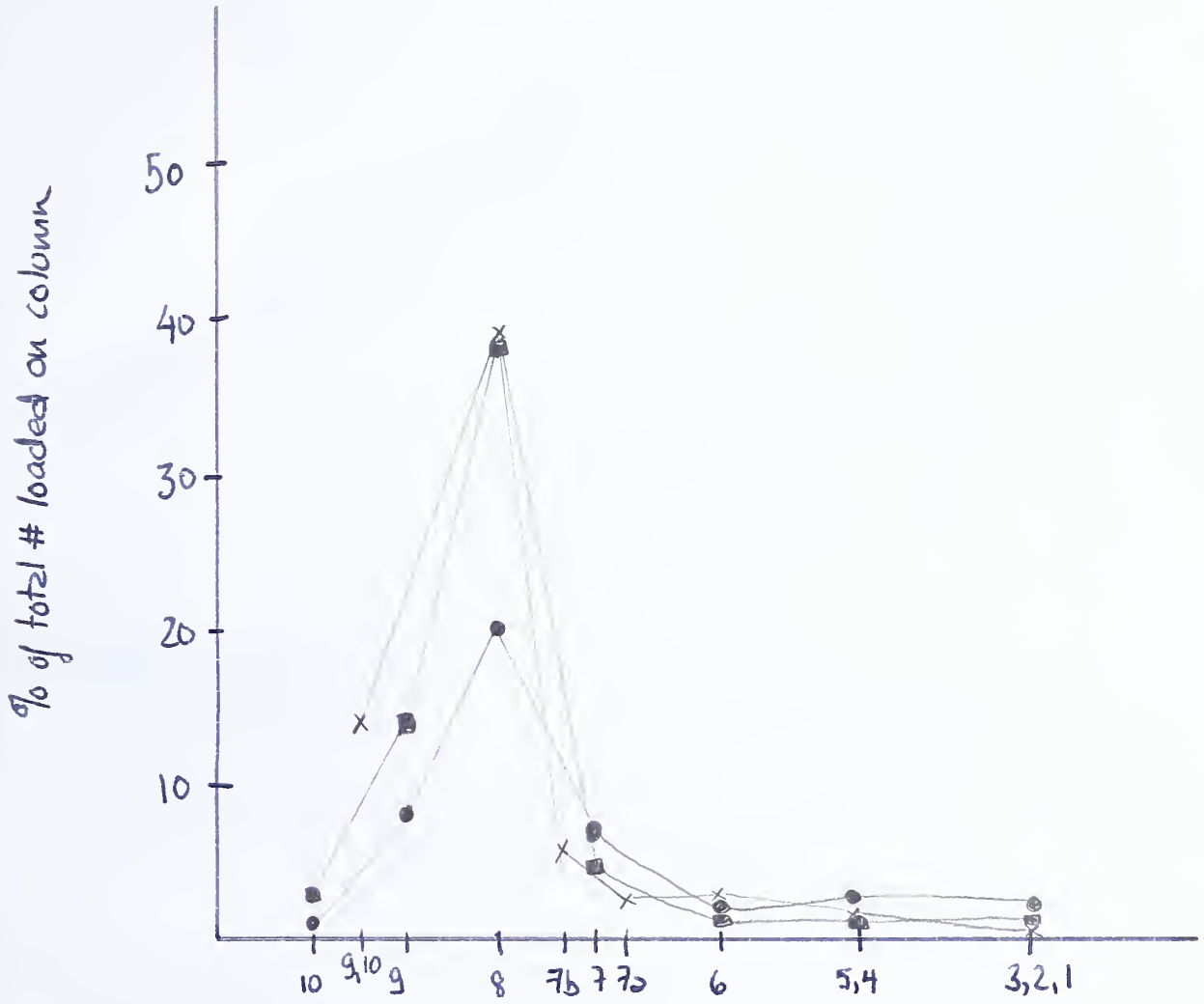
The relation between # of cells and response to antigen in microculture (0.1 ml) All cells were obtained from BALB/c mice.

Figure 2

	before nylon purification			after nylon purification			
# of cells	mean cpm ± SEM with stimulant	mean cpm ± SEM without stimulant	S.I.	mean cpm ± SEM with stimulant	mean cpm ± SEM without stimulant	S.I.	
10^6	26239 ± 2949	8604 ± 336	3.1	2310 ± 220	1783 ± 541	1.3	LPS
10^6	32367 ± 5229	8604 ± 336	3.8	15011 ± 425	1783 ± 541	8.4	PHA
5×10^5	7066 ± 2581	2779 ± 391	2.5	12540 ± 936	5206 ± 606	2.4	OVA

The effect of nylon purification on the response to LPS, PHA, and OVA (250ug) of lymphocytes obtained from BALB/C mice. The mice used for the experiments with LPS and PHA were unsensitized.

TABLE 3



● = exp #10 total # = 2.4×10^7

■ = exp #21 total # = 1.5×10^7

X = exp # MP10 total # = 2.2×10^7

The yield/fraction of cells separated by unit gravity sedimentation.

Figure 3


SIZE DISTRIBUTION GRAPH
wwsep.
4/22/76
MAXIMUM COUNT FOR ANY CHANNEL
% OF FULL SCALE
90
80
70
60
50
40
30
20
10
0
0=
CUBIC MICRONS
200

Figure 4a


COULTER MODEL I PLOTTER
SIZE DISTRIBUTION GRAPH
NAME LNC-Sens
NO 9,10 DATE 1/22/77
MAXIMUM COUNT FOR ANY CHANNEL
% OF FULL SCALE
90
80
70
60
50
40
30
20
10
200

Figure 4b


COULTER MODEL P PLOTTER
SIZE DISTRIBUTION GRAPH
NAME
NO.
DATE
MAXIMUM COUNT
FOR ANY CHANNEL
% OF FULL SCALE
90
80
70
60
50
40
30
CUBIC MICRONS
200

Figure 4c


COULTER MODEL P PLOTTER
SIZE DISTRIBUTION GRAPH
NAME
76
Sens CVC
DATE 11/22/76
MAXIMUM COUNT
FOR ANY CHANNEL
% OF FULL SCALE
90
80
70
60
50
40
30
20
10
0
0
CUBIC MICRONS
200

Figure 4d

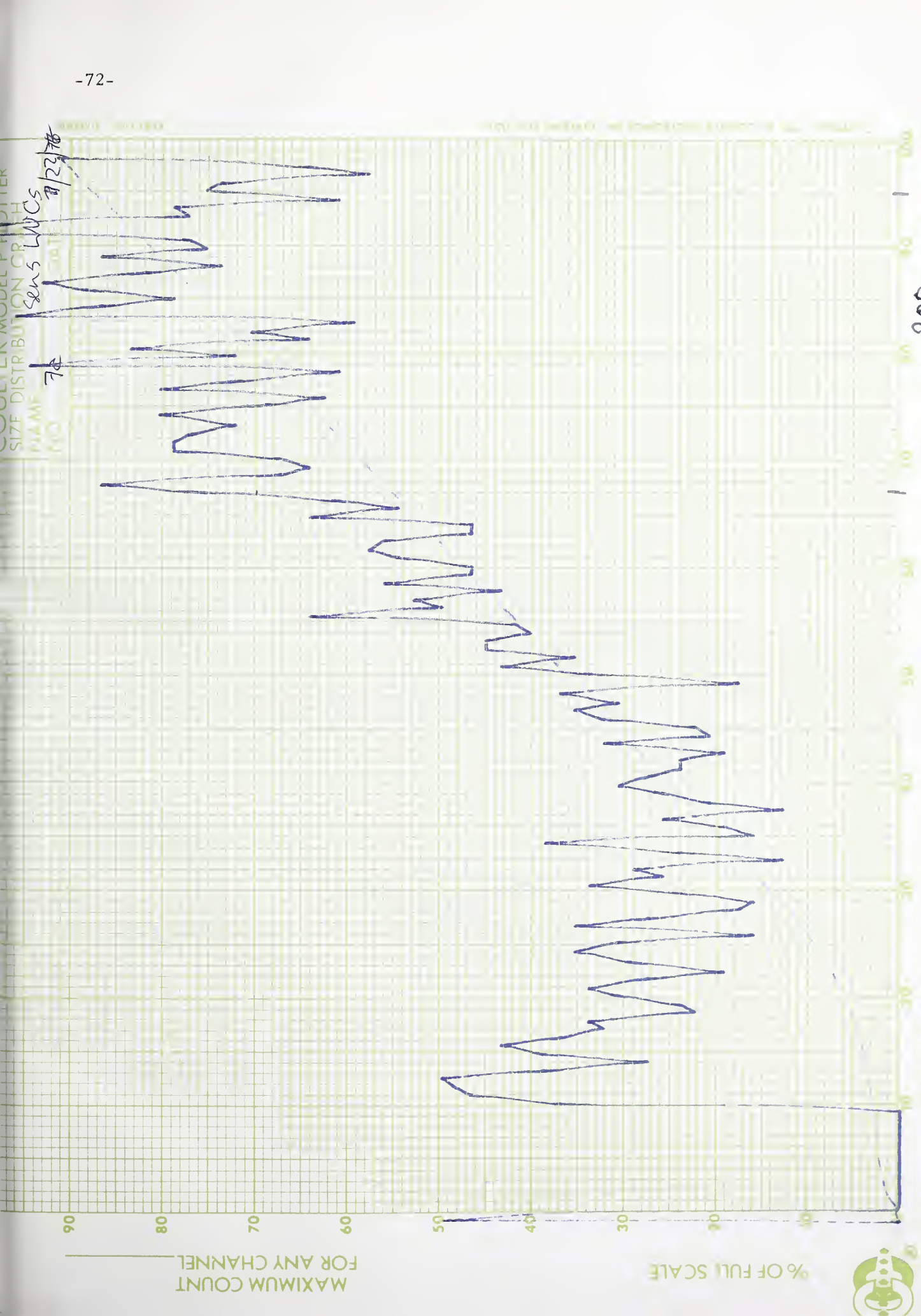
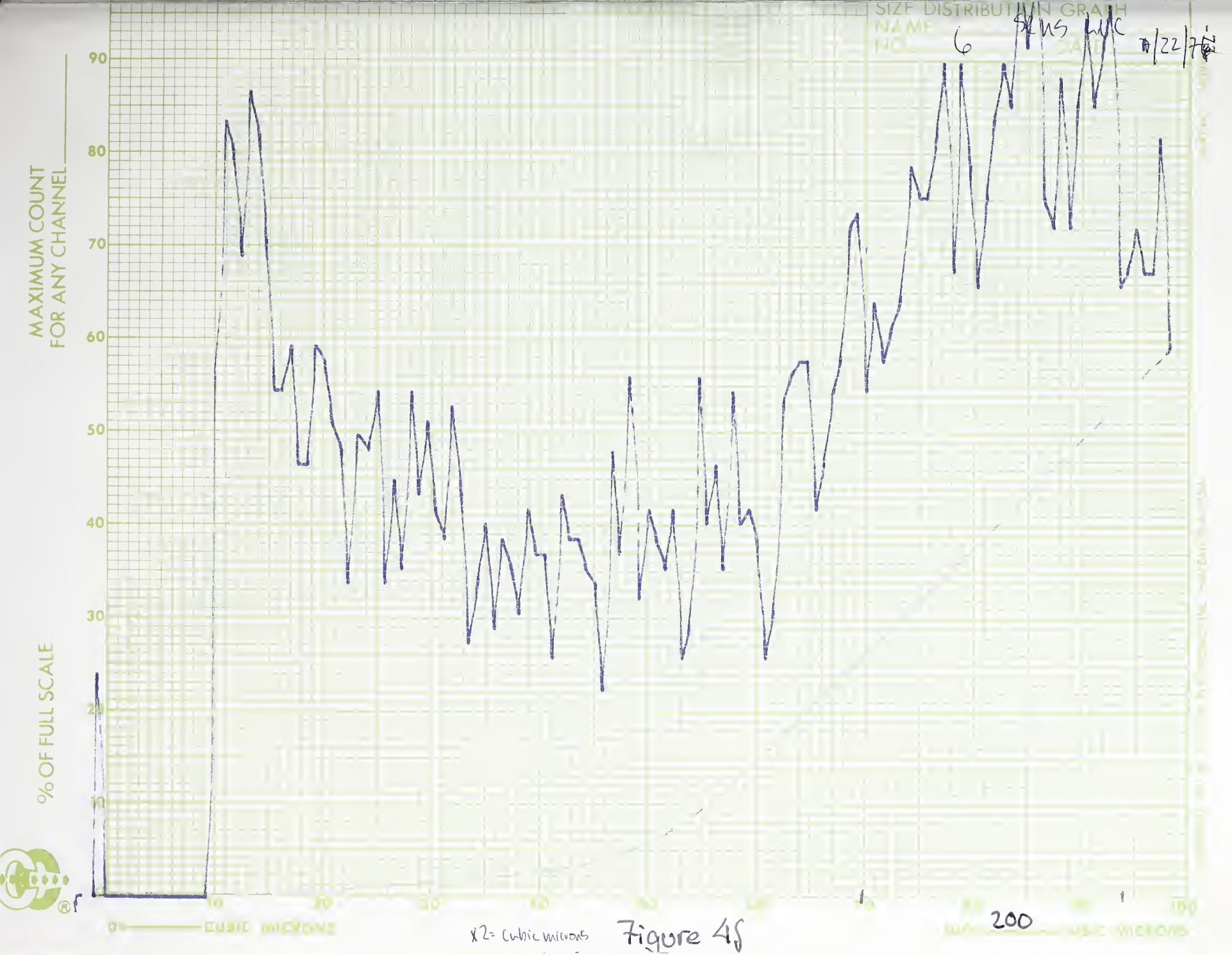

MAXIMUM COUNT
FOR ANY CHANNEL
% OF FULL SCALE
90
80
70
60
50
40
30
20
10
200

Figure 4e



X2= cubic microns

Figure 45

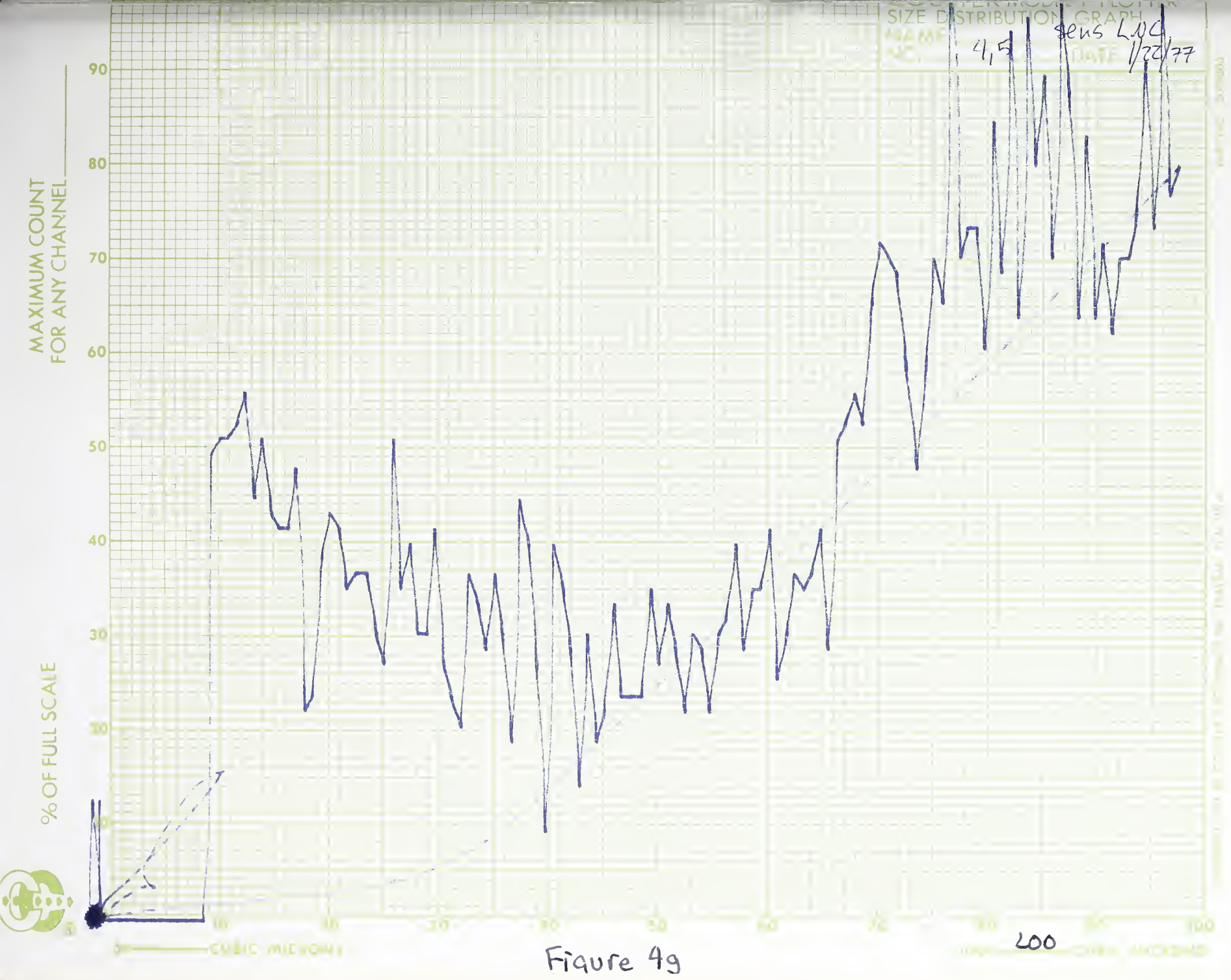

SIZE DISTRIBUTION GRAPH
4,5
Sens Log
1/22/77
MAXIMUM COUNT
FOR ANY CHANNEL
% OF FULL SCALE
90
80
70
60
50
40
30
20
10
0
CUBIC MICRONS
200

Figure 4g


SIZE DISTRIBUTION GRAPH
NAME C&WS LNC
NO 1,2,3
DATE 1/22/77
MAXIMUM COUNT FOR ANY CHANNEL
% OF FULL SCALE
90
80
70
60
50
40
30
20
10
0
200

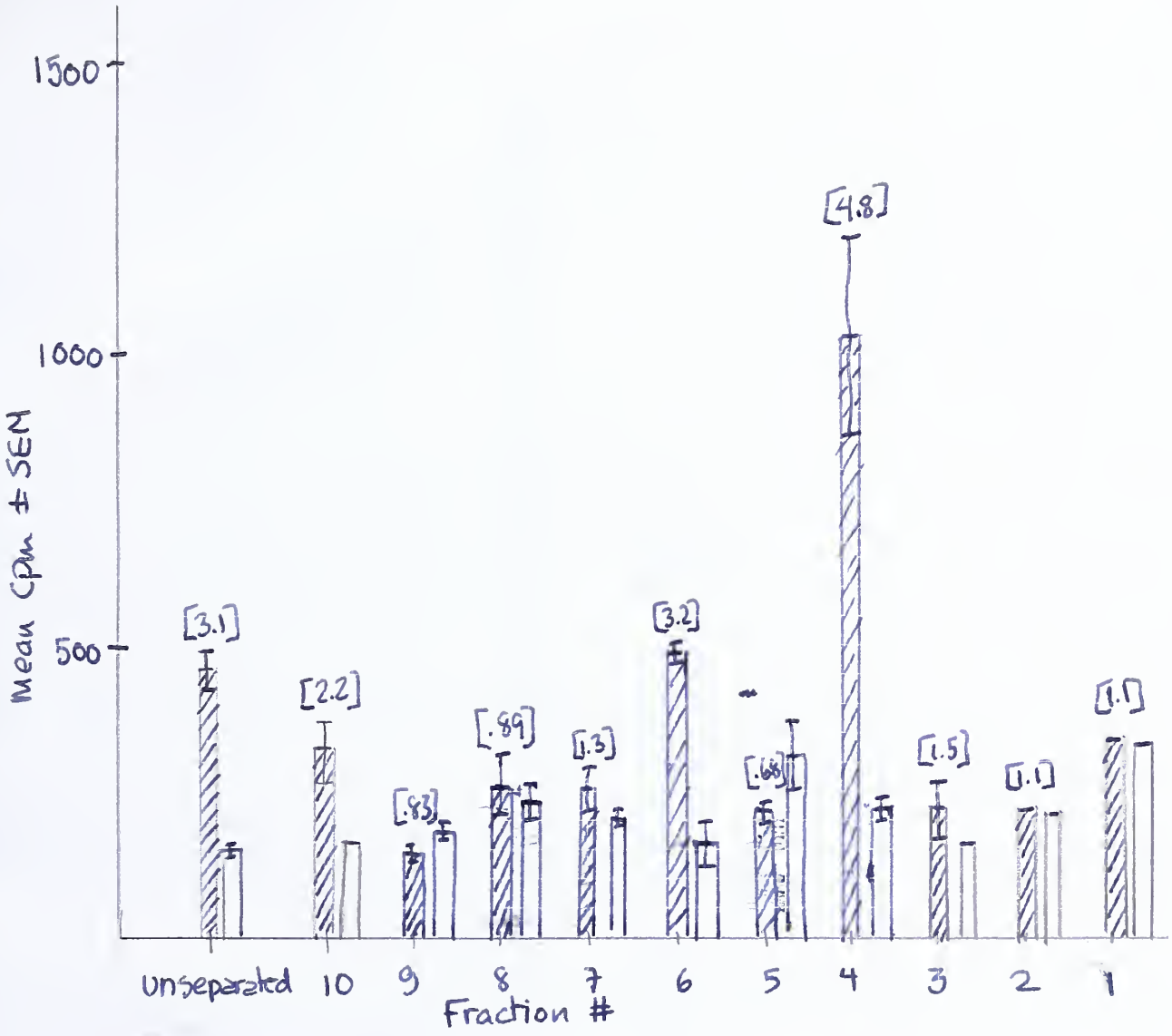
Figure 4h

Fraction	mean cell volume (μ^3)	mean cell diameter (μ)*	sedimentation velocity mm/hr
9, 10	100-120 20-44	5.84-6.21 3.42-4.45	0-4.50
8	99-132	5.83-6.42	4.5-6.75
7b	100-150	5.84-6.70	6.75-7.875
7a	134-190	6.45-7.24	7.875-9.0
6	140-188	6.54-7.22	9.0-11.25
4,5	150-200	6.70-7.37	11.25-15.75
1,2,3	110-130 20-40	6.04-6.38 3.42-4.45	15.75-22.50
UNSEPARATED	100-120	5.84-6.21	-

Mean cell volume, mean cell diameter, and sedimentation velocity of cells in fractions taken from a unit gravity sedimentation gradient. Cells are from C57Bl/6 mice.

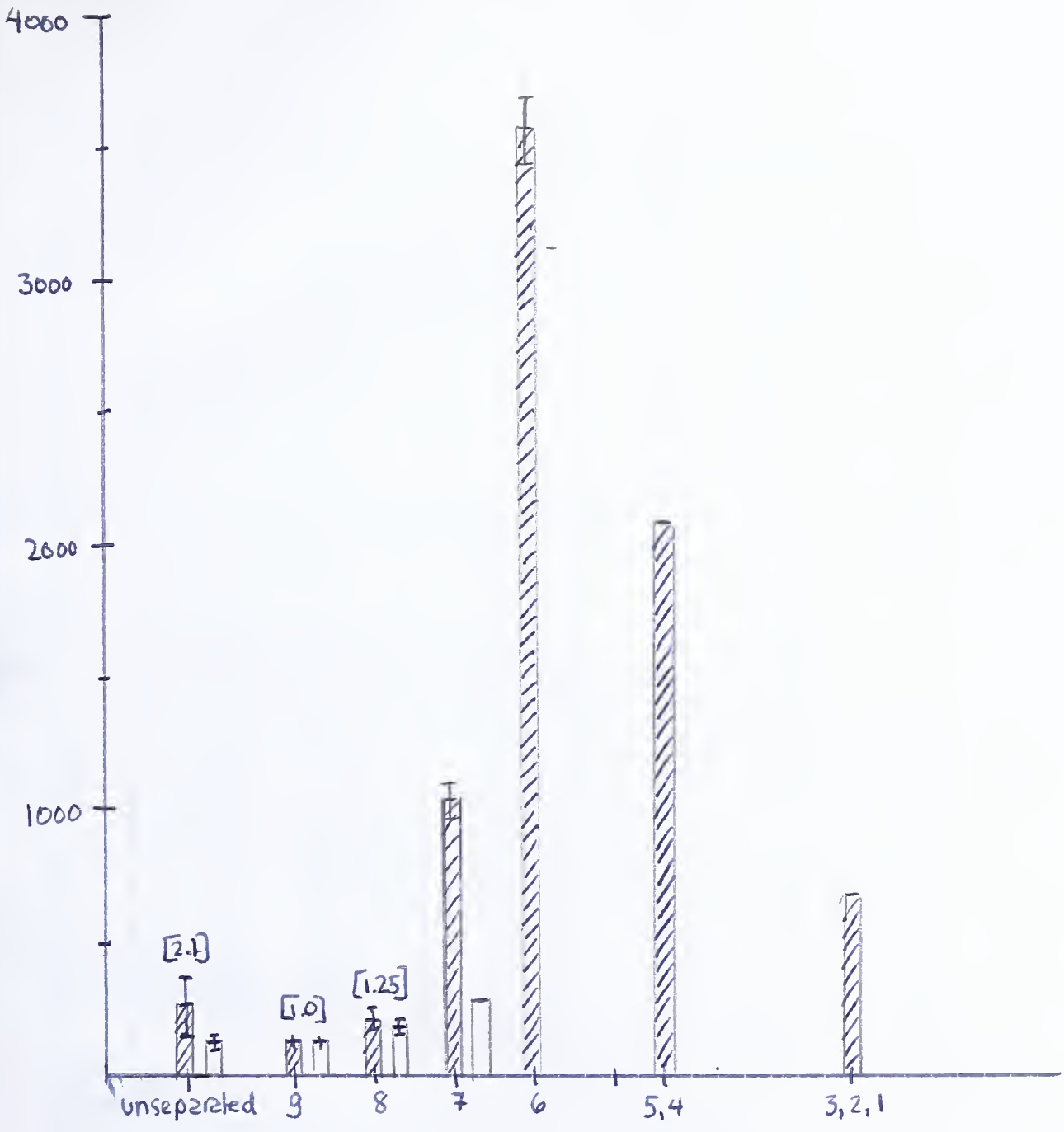
*Assuming that the cells are spherical.

TABLE 4



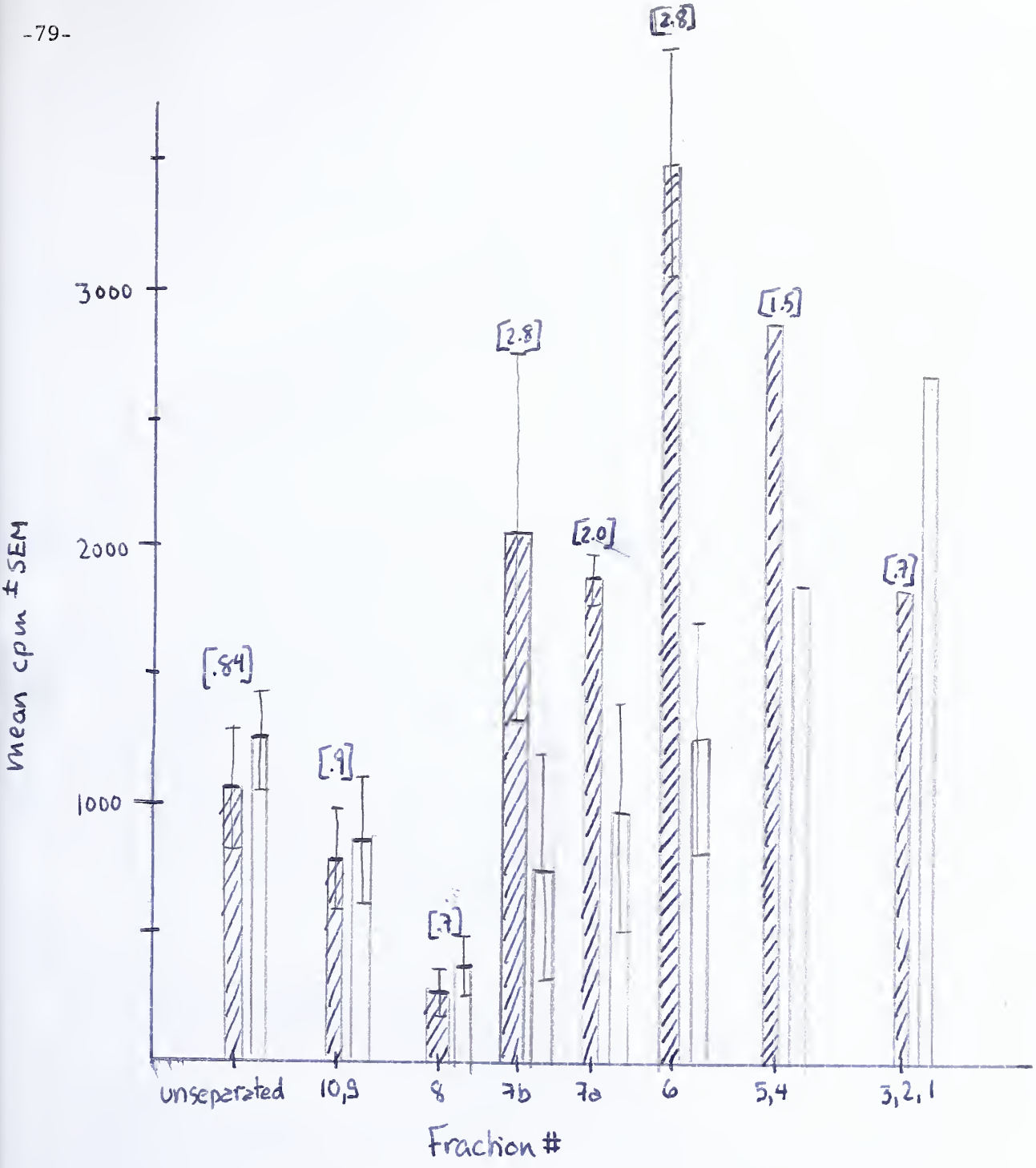
Separation of sensitized cells on a unit gravity sedimentation gradient. Cells were obtained from sensitized BALB/C mice and cultured at 5×10^4/ml culture.

Figure 5



Seperation of sensitized cells on a unit gravity sedimentation gradient. Cells were obtained from sensitized BALB/C mice and cultured at 5×10^4/.1ml culture.

Figure 6



Seperation of sensitized cells on a unit gravity sedimentation gradient. Cells were obtained from sensitized C57Bl/6 mice and cultured at 5×10^4 cells/.1 ml medium.

Figure 7

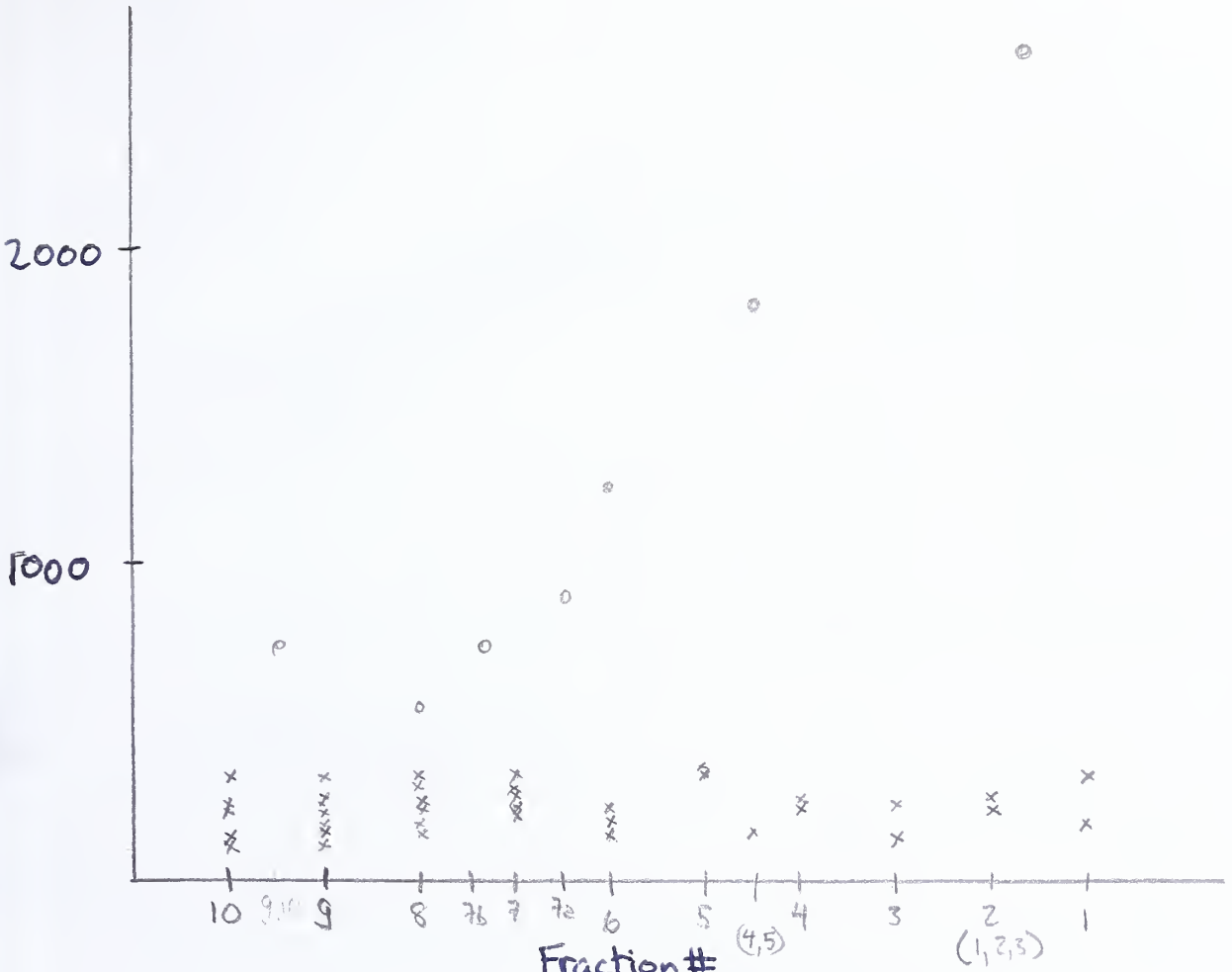

2000
1000
10
9
8
7b
7
7a
6
5
(4,5)
4
3
2
(1,2,3)
1
Fraction #
5×10⁴ cells/well
no antigen added
X-BALB
O-C57Bl
Unit Gravity Sedimentation
Background cpm

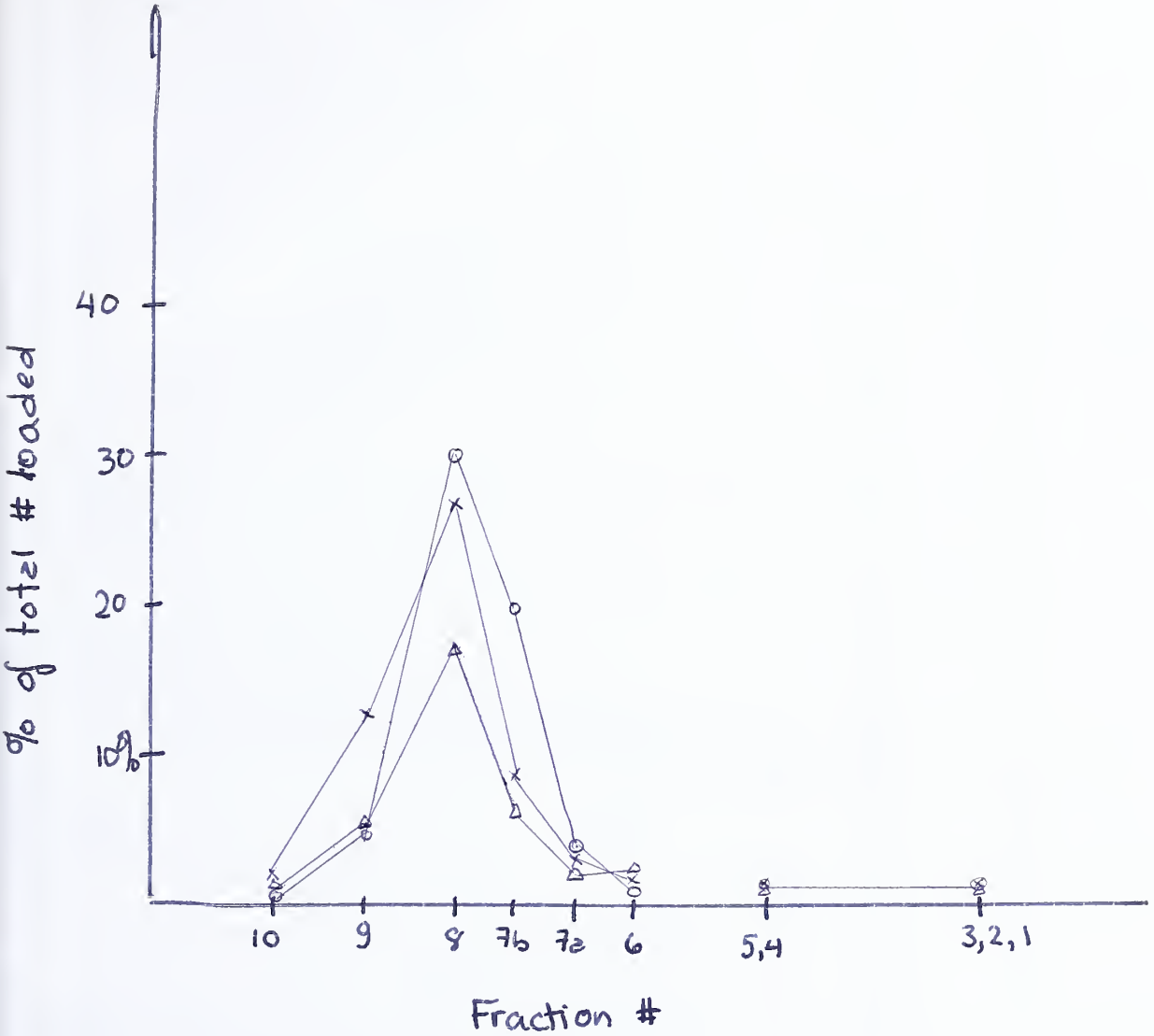
Figure 8

Fraction	% of total	small lymphocytes %	medium lymphocytes %	large lymphocytes %	macrophages %	sedimentation velocity mm/hr
unseparated	100	91	3	3	3	-
10	2.1	93	5	0	2	0-2.25
9	8.6	96	4	0	0	2.25-4.5
8	39.0	96	2.5	<1	1	4.5-6.75
7b	13.3	84	15	0	<1	6.75-7.875
7a	3.0	83	14	<1	3	7.875-9.0
6	1.6	63	26	1	10	9.0-11.25
4,5	.6	57	30	8	5	11.25-15.75
1,2,3	<.1	86	6	1	7	15.75-22.5

Differentials of cells taken from the various fractions after unit gravity sedimentation.

% of total represents the percent of the total cells initially loaded onto the sedimentation column.

TABLE 5



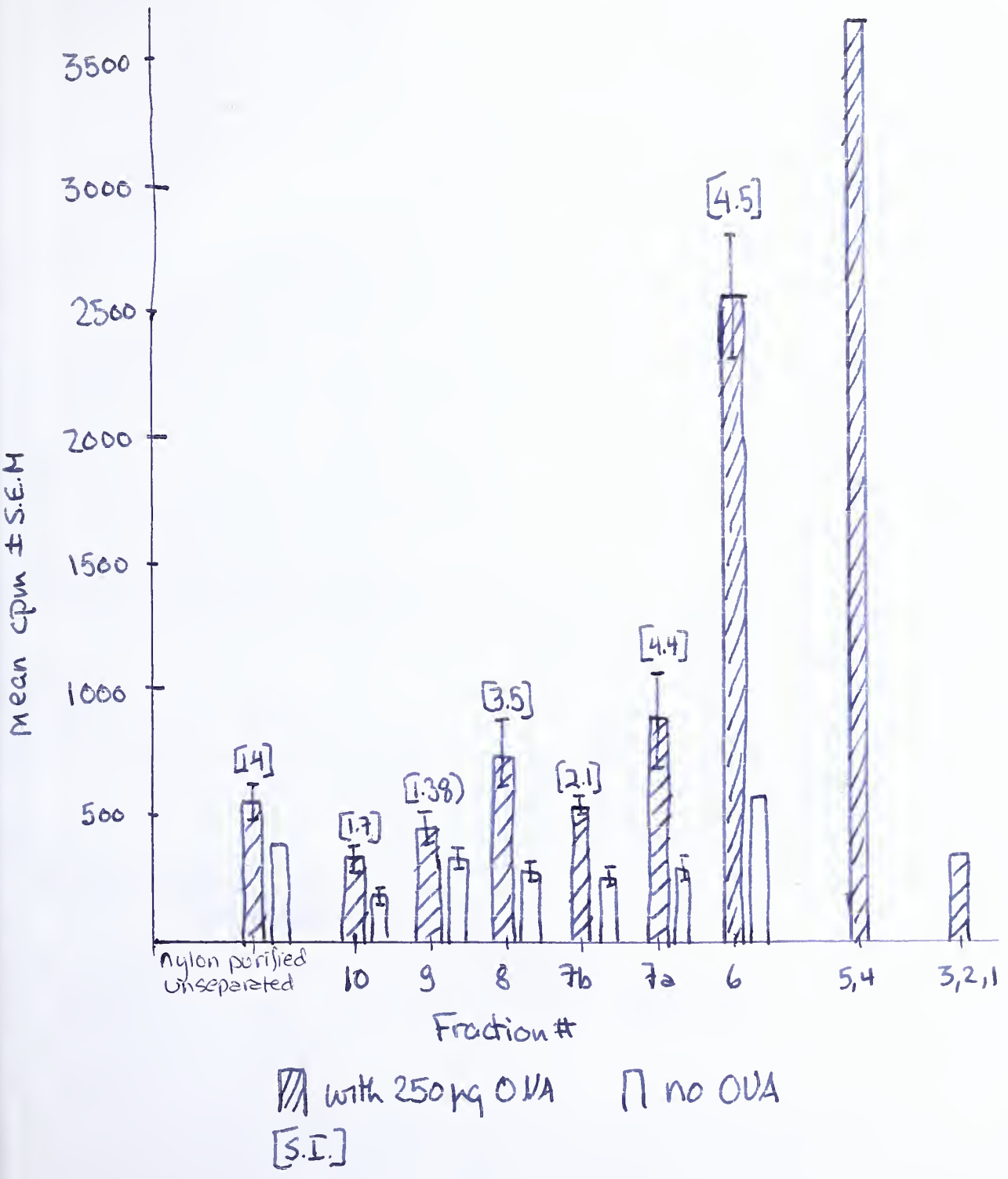
X = experiment #25 total = 2.4×10^7 - from BALB/c mice

O = experiment #27 total = 1.6×10^7 "

Δ = experiment #29 total = 2.3×10^7 from C57Bl/6 mice

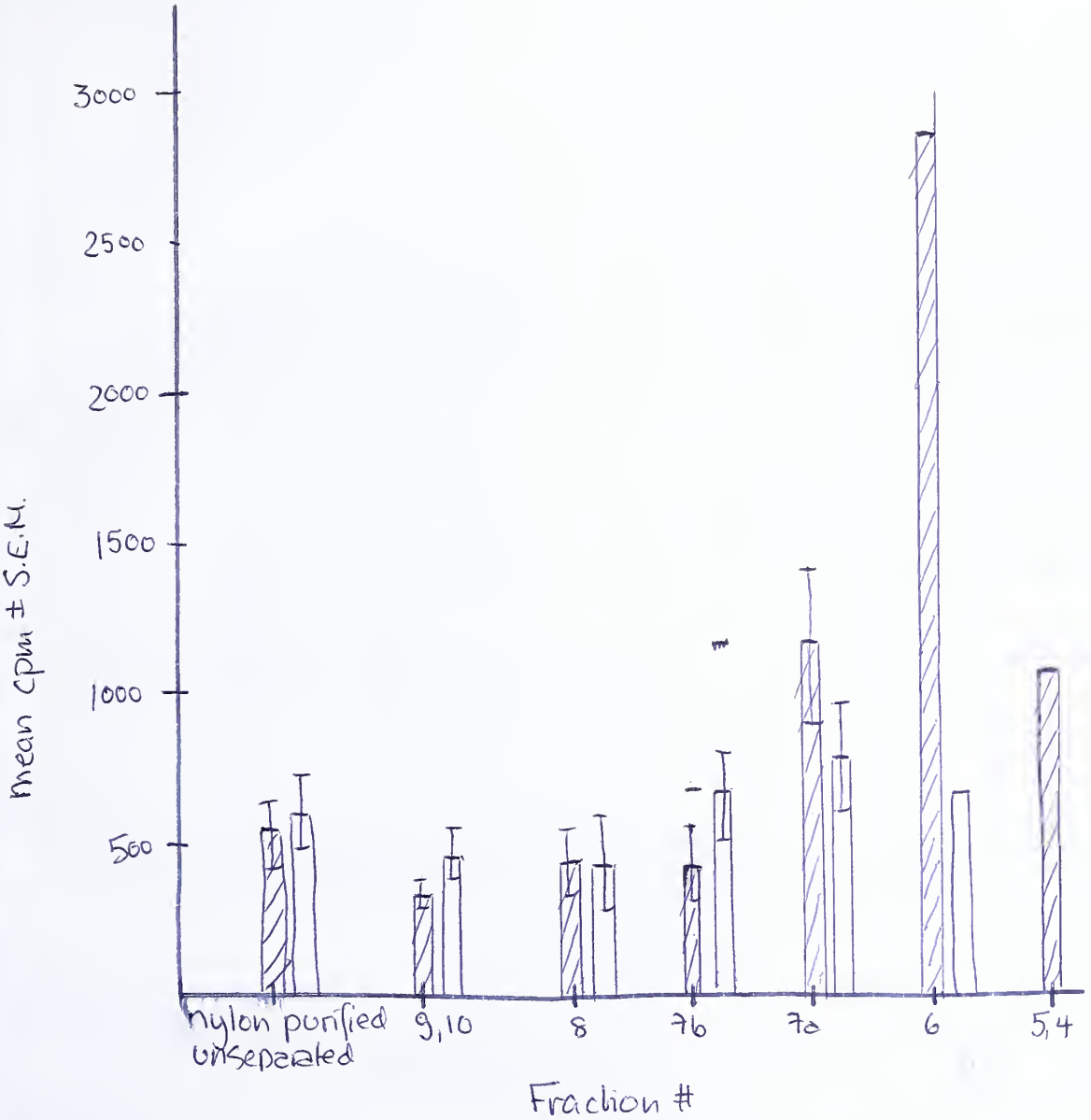
The yield/fraction of nylon-purified cells seperated by unit gravity sedimentation

Figure 89



The separation of nylon-purified cells by unit gravity sedimentation. Cells were obtained from sensitized BALB/c mice.

Figure 10



▨ - with 500 µg OVA ⊓ no OVA
[S.I.]

The separation of nylon purified cells on a unit gravity sedimentation gradient. The cells were obtained from C57Bl/6 mice sensitized with 100 µg OVA.

Figure 11.

Fraction	% of total	small lymphocytes %	medium lymphocytes %	large lymphocytes %	macrophages %	sedimentation velocity mm/hr
unseparated	100	94	5	1	0	-
10	1.9	93	6	1	<1	0-2.25
9	12.8	97	3	0	0	2.25-4.5
8	25.2	96	4	0	0	4.5-6.75
7b	8.5	95	5	0	0	6.75-7.875
7a	3.3	87	12	1	0	7.875-9.0
6	1.1	54	38	7	<1	9.0-11.25
1-5	.3	too few cells				11.25-22.5

Differentials of nylon purified cells taken from various fractions after unit gravity sedimentation.

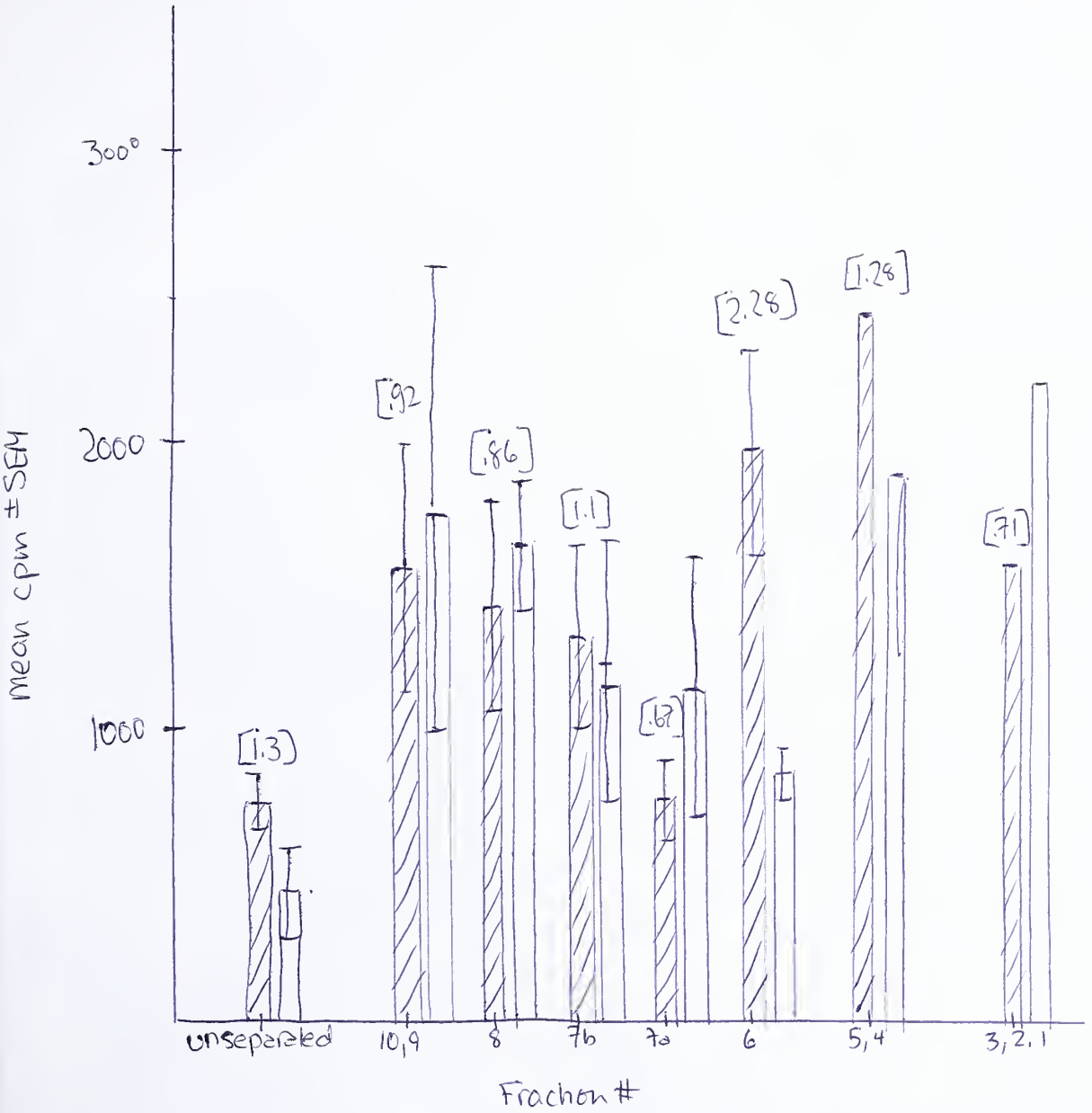
% of total represents the percent of the total cells initially loaded onto the sedimentation column.

TABLE 6

Fraction	small lymphocytes %	medium %	large %	macrophages %	sedimentation velocity mm/hr
10	94	5.5	.5	0	0-2.25
9	97.5	2.5	0	1	2.25-4.5
8	95	5	0	0	4.5-6.75
7b	slide poorly stained				6.75-7.875
7a	77	19	1.5	2.5	7.875-9.0
6	66	24	6	4	9.0-11.25
4,5	54	35	8.5	2.5	11.25-13.75
1,2,3	too few cells				13.75-22.5

Differentials of nylon purified cells taken from various fractions after unit gravity sedimentation.

TABLE 7



The effect of adding 5% pentoneal exudate cells on the separation of sensitized cells on a unit gravity sedimentation gradient. The cells were obtained from sensitized C57Bl/6 mice.

Figure 12

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